Click Chemistry Reactions in Medicinal Chemistry: Applications of the 1,3-dipolar Cycloaddition Between Azides and Alkynes

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Abstract: In recent years, there has been an ever-increasing need for rapid reactions that meet the three main criteria of an ideal synthesis: efficiency, versatility, and selectivity. Such reactions would allow medicinal chemistry to keep pace with the multitude of information derived from modern biological screening techniques. The present review describes one of these reactions, the 1,3-dipolar cycloaddition (“click-reaction”) between azides and alkynes catalyzed by copper (I) salts. The simplicity of this reaction and the ease of purification of the resulting products have opened new opportunities in generating vast arrays of compounds with biological potential. The present review will outline the accomplishments of this strategy achieved so far and outline some of medicinal chemistry applications in which click-chemistry might be relevant in the future.

Key words: click chemistry; 1,3-dipolar cycloaddition; azide; alkyne; copper; triazole; bioconjugation

1. INTRODUCTION

The main target in medicinal chemistry is to synthesize compounds or libraries of compounds during the process of drug discovery or lead optimization, and for this reason, this field is particularly attracted to synthetic methodologies that allow rapid construction of molecules. The identification of such rapid synthetic strategies should allow the medicinal chemist to assemble a large number of
biologically active compounds in a very short period of time speeding up the process of discovery and lead optimization. Yet, alongside being rapid, the key features of the ideal synthesis are efficiency, versatility and selectivity. In 2001, the term “click chemistry” was first coined to describe reactions defined by a set of stringent criteria: “The reaction must be modular, wide in scope, give very high yields, generate only inoffensive by-products that can be removed by non-chromatographic methods, and be stereospecific (but not necessarily enantioselective). The required process characteristics include simple reaction conditions, readily available starting materials and reagents, the use of no solvents or a solvent that is benign (such as water) or easily removed, and simple product isolation.”

To date, the most popular reaction that has been adapted to fulfill these criteria is the 1,3-dipolar cycloaddition, also known as Huisgen cycloaddition, between an azide and a terminal alkyne affording the 1,2,3-triazole moiety. This reaction was discovered at the beginning of the 20th century, but the potential of this reaction and its mechanism were only unveiled in the 1960s by Huisgen et al.3

The potential of this reaction is very high, since alkyne and azide components can be incorporated into a wide range of substituents. Yet, as the directing effect of the substituents is usually weak, for more than 40 years this reaction suffered from a lack of selectivity yielding a mixture of the 1,4- and the 1,5-regioisomers. Furthermore, this transformation requires heating and long reaction times to go to completion and the two regioisomers are at times laborious to separate using classical chromatographic procedures. In 2002, two groups independently reported that copper (I) salts were able to accelerate this reaction by up to 10 million times. More importantly, at room temperature or with only moderate heating, the copper catalyst directs the formation of only one of the two regioisomers, namely the 1,4-regioisomer (Fig. 1). Furthermore, the conditions described in these manuscripts fulfilled the click chemistry criteria stated above, converting a very good reaction into the “cream of the crop.”

1,4-Disubstituted triazoles are not a novelty in medicinal chemistry. Indeed, more than 7,000 1,4-disubstituted 1H-1,2,3-triazole compounds had been reported before the discovery of the copper catalyzed click reaction. Triazoles have been shown to possess a number of desirable features in the context of medicinal chemistry. For example, triazoles are stable to acid and basic hydrolysis and reductive and oxidative conditions, indicative of a high aromatic stabilization. At the same time, this heterocycle has a high dipole moment (about 5 D) and might be also able to participate actively in hydrogen bond formation as well as in dipole–dipole and π stacking interactions. Finally, this moiety is relatively resistant to metabolic degradation.

Among the best-known examples of triazole-containing structures is tazobactam, a β-lactamase inhibitor which is marketed in combination with the broad spectrum antibiotic piperacillin. Indeed, when first described, tazobactam (1) and related triazole-containing compounds (e.g., BRL-42715) (Fig. 2) turned out to be potent β-lactamase inhibitors with higher potency than clavulanic acid and sulbactam, and the triazole ring appears to play a pivotal role for its potency. In the antibiotics field, triazoles have been also used to improve pharmacokinetic properties of the desired drug. For example, cephalosporins endowed with good oral availability were obtained linking the triazole moiety to the cephalosporin core (3) (Fig. 2). Indeed, it is not just antibiotics which benefit from the triazole ring. Analogues of the pyrimidine nucleosides have been studied extensively as antiviral agents and a triazole moiety has been used as surrogate of the pyrimidine ring to give compounds (e.g., 4; Fig. 2) which were reported to have both antiviral and cytotoxic properties. Similar analogues, bearing a good leaving group (5) (Fig. 2), were synthesized to be used as alkylating agents in cancer chemotherapy. Last, triazoles have been employed to attempt to generate functional, non-classical bioisosteres to substitute potentially labile functional groups (e.g., esters, amides) with heterocyclic rings which are able to mimic the same electrostatic potential maps (arenology for simplicity, 1,2,3-triazole will be shortened to triazole in the manuscript.

aSciFinder Scholar search for 1,4 disubstituted 1,2,3-triazoles up to year 2002.
principle). The triazole moiety, for instance, has been shown to act as a non-classical bioisostere of an ester group in arecoline, a muscarinic agonist, although in this case, other heterocycles have proven to be better mimetics.

A. Solvent, Catalysts and Additives in the 1,3-Dipolar Cycloaddition

As mentioned above, the use of copper as a catalyst rejuvenated the Huisgen reaction. The standard catalytic system uses copper (II) salts (e.g., copper sulfate pentahydrate or copper acetate) in the presence of a reducing agent, such as sodium ascorbate or metallic copper. This constantly reduces copper (II) to copper (I) maintaining significantly high levels of the catalytic species. A mixture of tert-butanol and water is used as solvent, as under these conditions it is not necessary to use a base to generate the copper acetylide species. It is important to stress this solvent can also be used for lipophilic compounds. When aqueous conditions cannot be used, organic solvents (e.g., tetrahydrofuran, toluene, dichloromethane, acetonitrile) in the presence of stoichiometric amount of copper (I) salts (e.g., CuI, Cu(CH3CN)4PF6, CuBr(PPh3)4 or CuIP(OEt)3) and an excess of a base, usually a tertiary amine (e.g., TEA, DIPEA) can be used.

The improvement of better copper-based catalysts is still ongoing. For example, very recently, copper-in-charcoal, has been developed as a simple, inexpensive, and efficient heterogeneous catalyst.

Figure 1. The 1,3-dipolar cycloaddition between azides and alkynes.

Figure 2. Examples of 1,4-disubstituted triazoles synthesized before the advent of the Cu-catalyzed azide-alkyne cycloaddition.
catalyst for triazole formation. The potential advantages of this new catalyst are many fold. Indeed, it can be used with a large variety of solvents, it is easily removable by filtration and it has been reused in at least two additional cycloadditions without loss of activity. It has also been demonstrated that its use reduces or abolishes the contamination of the product by copper. Finally the use of a base is not strictly necessary to effectively catalyze this cycloaddition.

The “canonical” 1,3-dipolar cycloaddition click chemistry reaction between azides and alkynes yields only the 1,4-disubstituted triazole ring and efforts have therefore been made to expand the applicability of this reaction by developing an orthogonal cycloaddition to give the 1,5-disubstituted product. This would make this click chemistry reaction a true regiocomplementary reaction, yielding the desired regioisomer according to the conditions used. Indeed, some papers have appeared in the past few years where the 1,5-disubstituted triazole products were obtained as prevalent isomers. For instance, it was reported that this regioisomer can be prepared by the reaction of bromomagnesium acetylenes and organic azides and in a recent paper this strategy was successfully utilized to prepare a set of triazole-based monophosphyne ligands (clickPhos). On the other hand, the transformation of the alkyne group into the bromomagnesium acetylene with the Grignard reagent is troublesome with functionalized (e.g., esters, enolisable substrates, alcohols) substrates and thereby leads to the loss of the versatility feature of click reactions.

Substituted trimethylsilyl acetylenes have also reported to direct the cycloaddition affording the 1,5-regioisomers. The trimethylsilyl group probably controls the selectivity by a combination of two factors: the ability to stabilize a positive charge on acetylene at the β-carbon position in the transition state and the steric hindrance that precludes the reverse orientation. However, few reports have shown the success of this strategy and a systematic use of this reaction has never been attempted. The conditions of this reaction are not as simple as those of the “canonical” 1,3-dipolar cycloaddition click chemistry reaction and this might explain its modest use. Yet, in our opinion it would be interesting to investigate its universality.

In 2005, the possibility to direct the 1,3-dipolar cycloaddition between alkynes and azides to yield the 1,5-disubstituted triazole, using the Cp*RuCl(PPh3)2 catalyst, was reported (Fig. 1). It is noteworthy that this catalyst, unlike copper, allows the reaction between internal alkynes and azides, and a systematic study using asymmetric internal alkynes has been published.

Although copper appears to be the perfect catalyst and the procedures have now been well established, the diverse potential uses (see below) that have been postulated for the 1,3-dipolar cycloaddition between azides and alkynes still allow for further investigation on conditions that might favor particular applications. To this end, a number of additives that might allow the reduction of the copper concentrations or increase the efficiency of the reaction have been described, such as the (tris-(benzyl-triazolylmethyl)amine) (TBTA) (6), triethylamine hydrochloride (7) and the water soluble sulfonated bathopenantroline (8) (Fig. 3). These additives appear very promising, for example, for the bioconjugation of particularly expensive or precious substrates.
The use of the 1,3-dipolar cycloaddition reaction between azides and alkynes, in combination with classical organic chemistry, has led to the synthesis of a huge variety of synthetic structures in a very short time with different and, at times, outstanding biological properties. This review will focus only on the medicinal chemistry applications of the copper-catalyzed azide-alkyne cycloaddition (CuAAC) to yield triazole derivatives, while mechanistic considerations, as well as the application of the 1,3-dipolar cycloaddition between azides and alkynes in other fields of research will not be covered. The present review has as its main aim the description of the possible advantages of click chemistry in traditional applications of medicinal chemistry, via the use of selected examples.

2. TRIAZOLE RINGS AS BIOISOSTERES

Since CuAAC has made the insertion of the triazole ring relatively easy, it is important to establish the bioisosteric potential of this moiety, to broaden the use of the reaction in the improvement of hit compounds.

There are important clues that suggest that the triazole group displays structural similarity with the amide bond, mimicking a Z or an E-amide bond depending on its substitution pattern. Thus, the 1,4-disubstituted triazole moiety shows similarity with a Z-amide bond: the lone pair of the 3-nitrogen mimics the one of the carbonyl oxygen of the amide bond, the polarized C(5)-H bond can act as a hydrogen bonding donor, just like the amide N–H bond, and the electrophilic and polarized 4-carbon is electronically similar to the carbonyl carbon. Since the overall dipolar moment of the triazole system is larger than that of the amide bond, its hydrogen bonding donor and acceptor properties are more marked than those of an amide bond, with an enhanced peptide mimicry. The major structural difference between a triazole and a Z-amide is the distance between the substituents, linked by two atoms in the amide, and by three atoms in the triazole ring, with an overall increase of about 1.1 Å. On the other hand, the 1,5-substitution pattern mimics the E-amide bond. In this case, the link between the substituents is identical in terms of the atoms involved and the relative position of the hydrogen bonding donor and acceptor sites is also similar. Yet, there are some differences in atom polarization, as the electrophilic carbonyl carbon is now replaced by a negatively polarized nitrogen atom (Scheme 1). The value of triazole as an amide bond surrogate (the oxygen lone pair, the acidic N–H bond and the polarized carbonyl carbon) in peptidomimetic structures has also been suggested experimentally.

Molecular modeling, furthermore, has also suggested that a 1,4-disubstituted triazole could be consistent with the geometry of a β-turn. Experimentally, a β-turn was achieved by using three-carbon linkers in a simple model peptide system (9) (Fig. 4). The use of triazoles in the field of peptidomimetics is further strengthened by a report that showed that triazole ε²-amino acids were effective surrogates of dipeptides in α-helical structures (10) (Fig. 4). Furthermore, 1,4-disubstituted triazole oligomers have been suggested to closely mimic the structure of β-strands (11).

Besides the potential of triazoles in mimicking the amide of the peptide bond, it has also been predicted that triazoles might act as bioisosteres of the acyl-phosphate and trans-olefinic moieties. The effort to develop inhibitors of enzymes involved in the synthesis of siderophores (iron-chelators...
required for growth and virulence) in *Mycobacterium tuberculosis* led to the attempt to substitute the acyl-phosphate in an intermediate substrate (12) with a triazole to generate intermediate mimics. Since the acyl-phosphate group on the intermediate is fundamental for the second phase of the reaction, as it is a good leaving group, the authors attempted to substitute it with the stable triazole via click chemistry (13) (Fig. 5). The choice of this heterocycle was due to the presence of the critical four-atom spacer and multiple H-bonding sites. In this case, the 1,4-disubstituted triazole analogue was ineffective as an inhibitor and molecular modeling suggested that this lack of activity was due to the planar geometry of the triazole, which prevented it from acting as a hydrogen bond acceptor.38

The substitution of a trans-olefinic group with a triazole in resveratrol proved to be more rewarding. The idea was supported by molecular modeling calculations (MM2), that suggested that this modification did not alter the spatial positioning of the phenolic hydroxyls that determine the biological activity of this phytoalexin. Resveratrol (14) has been demonstrated to have a number of potentially interesting biological activities, but its usefulness is limited as it exerts most of its biological effects at high micromolar concentrations, making it difficult to ascertain which are the targets responsible for the single effects. A parallel combinatorial approach was used to synthesize triazole analogues via click chemistry in the hope of finding resveratrol analogues that maintained some, but not all, properties of the parent compound. Seventy-two compounds were submitted to biological screening and five lead compounds were identified (Fig. 5).39 The effective trans-olefinic/
1,4-disubstituted triazole replacement is not limited to resveratrol, as it was possible to maintain estrogenic activity in diethylstilbestrol analogues.\textsuperscript{40} Grimm’s bioisosteric rule would suggest that obvious candidates for bioisosteric substitution with triazoles should be five-membered rings containing two nitrogen atoms. Surprisingly, the literature does not present as many attempts at this as would be expected. A good example of the success of this substitution is represented by work on fipronil (15) (Fig. 6), an insecticide that acts both as a non-competitive antagonist on insect GABA receptors and as a blocker of insect glutamate-gated chlorine channels. A library of thirty 1-phenyl-1\textit{H}-1,2,3-triazole derivatives was synthesized replacing the pyrazole ring with a triazole via CuAAC. Some 1,5-regioisomers were also generated using the thermal protocol. Strikingly, several 1,4 regioisomers, but not their 1,5 counterparts, were more potent competitive inhibitors compared to fipronil. The potency and selectivity were determined by substituents, strengthening the idea that triazole is a Grimm’s bioisostere of pyrazole.

\textbf{Figure 5.} Attempts to use triazole as a bioisostere of acylphosphate and trans-olefinic moieties.

\textbf{Figure 6.} Attempts to use triazole as a bioisostere of heterocycles.
This claim is reinforced by experiments on carbocyclic nucleosides. To discover more selective analogues, derivatives of neplanocin A (16) (Fig. 6) were synthesized, replacing the adenine with other heterocyclic moieties. In particular, the 1,4-disubstituted triazole derivative (17), prepared with the CuAAC procedure, had the most potent antiviral activity against vaccinia virus among the five-membered rings tested (Fig. 6).

The bioisosteric replacement with a triazole via CuAAC should, in our opinion, move from a rarity to the lab routine. Indeed, it is likely that amides, trans-olefinic moieties and the rings described above are not the only possible candidates and this issue should be investigated.

3. CuAAC AS AN AID TO RAPID SYNTHESIS

A. Homodimers, Heterodimers and Fragment-Based Drug Discovery

The simplicity and tolerance of the CuAAC reaction, together with the relative inertness of the triazole ring, suggests that this protocol can be exploited to link two or more molecular entities. For example, this reaction can be used for the generation of dimers, chimeras and multivalent drugs. The triazole in this case could be seen as an inactive linker or spacer, although it cannot be excluded that, at times, it may act as a biological entity on its own. Homodimers have at times proven to be a successful strategy to increase the potency of compounds, since the binding of one molecule should bring the second closer to its target. The first attempt at using click chemistry with these assumptions was on Vitamin D3 derivatives. The CuAAC reaction was performed on the side chain of Vitamin D3, giving the desired 1,4-disubstituted triazole dimers (e.g., 18) in high yield and total regioselective control (Fig. 7). Although the reaction was successful, the dimer turned out to be inactive biologically (i.e., no antiproliferative activity). In a more successful example, dimers of daunorubicin were synthesized using click chemistry and the distance between the two monomers was varied via different linkers containing triazole (e.g., 19) (Fig. 7). The rationale was that the dimerization might prove useful to give more potent DNA intercalators. In fact, it is well known that daunorubicin bind DNA in a stochiometric ratio of 2:1. The products were tested on the K562 tumoral cell line and the results pointed out that the distance between the two monomers, given by the length and the flexibility of the linker, is crucial to the resulting activity, as one would expect.

An extension of the dimer/polymer idea is the use of linkers to generate chimeric drugs bearing two distinct activities. For instance, the CuAAC reaction was used to assemble a multivalent antibiotic with linezolid and a macrolide or vancomycin and a cephalosporin (Fig. 7). Recent work on kabiramide C (22) (Fig. 8), a natural compound that has been shown to interact with actin, shows the potential of CuAAC to aid the synthesis of natural product analogues. In order to synthesize new semi-synthetic analogues of this macrocycle, Petchprayoon et al. sought to introduce an amino group in place of the hydroxyl group in position 7. In this manner, it would have been possible to synthesize rapidly a number of derivatives using activated carboxylic esters. The complexity of the molecule hampered the formation of the desired amino derivative, and the Authors turned their attention to the synthesis of different amino derivatives of kabiramide C bearing a triazole as linker group. Indeed, compound (23), was shown to interact strongly with G-actin and toxicity assays on human cervix carcinoma cells revealed that this molecule was as cytotoxic as kabiramide C (Fig. 8). The triazole linker has also been used to generate bidentate inhibitors, where the molecule interacts with two different binding sites of the same target. This has been employed to identify protein tyrosine phosphatase (PTP) inhibitors. In this instance, N-phenyloxamic acid was chosen as a core group, as it was demonstrated that it could bind the primary site of PTP1. As potential peripheral groups, aromatic rings with different polarity and alkyl linkers of different lengths were chosen. A library of 66 compounds was synthesized in water and the triazoles obtained were directly screened.
as inhibitors without purification. Six hits were identified and one (24) was shown to have activity in
the micromolar range and to be more selective toward one of the tyrosine phosphatase subtypes
(PTP1B; Fig. 9).49

A similar idea was exploited to identify selective matrix metalloprotease (MMP) inhibitors. In
this case, the “warheads” were eight different substituted succinyl hydroxamates (well known to
interact with a zinc ion present in the active site of the enzyme) bearing a terminal alkyne. These were

Figure 7. Selected examples of homodimers and heterodimers joined by triazole rings.

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Figure 8. Kabiramid triazole-analogue.

Figure 9. Examples of CuAAC in fragment-based drug discovery.

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coupled with 12 different hydrophobic azides via a parallel combinatorial approach. Two of them (25 and 26) were inhibitors of MMP 7 with activity in the micromolar range (Fig. 9).  

A library of compounds with a hydroxyethylamine core, which is known to be a bioisostere of amide groups, was coupled to a series of different alkynes via triazole formation in an attempt to generate HIV protease inhibitors. After the preparation of the azide derivatives (27 and 28) (Fig. 10), 50 different alkyne moieties were added in the presence of copper sulfate/copper turnings using a parallel combinatorial approach. After incubation for 48 hr, the mixture was analyzed via LC–MS in order to verify the formation of the desired adduct. As the reaction mixture appeared clean, direct biological evaluation was made without purification. Two compounds (e.g., 29) were found to be potent as HIV-1 protease inhibitors in the nanomolar range. Indeed CuAAC has found another application in the field of HIV research. Addition of a triazole derivative to a peptide known to interact with gp120 afforded an inhibitor (30) which blocked the interaction with cell-surface receptors of the host tissue with a potency two orders of magnitude greater than the original inhibitor (Fig. 10).  

Another exciting example comes from fucosyltransferase, an enzyme which catalyzes the transfer of L-fucose from guanosine diphosphate β-L-fucose to the corresponding glycoconjugate acceptor. Fucosylated saccharides play an important role in inflammation, thereby making inhibitors of this enzyme a potential target for anti-inflammatory drugs. Using the CuAAC approach, a new inhibitor of this enzyme was identified in a very simple and rapid manner. In detail, the authors generated a library of 85 triazole derivatives by reacting a GDP-alkyne with different hydrophobic azides in water, without the use of protecting groups. Water can act as a protecting group itself and,
indeed, the troublesome dianionic phosphate linkage gave no problem. The obtained compounds were so pure that they were screened directly for biological activity, yielding three hit compounds. One of these proved to be a competitive inhibitor with a $K_i$ of 62 nM (31) (Fig. 11). Yet, it should be noted that this direct approach, where the reaction mixture is directly screened in the biological assay (a procedure defined “microtiter plate based chemistry and in situ screening”), is not always applicable and requires some precautions. For example, reagents, buffers, and the copper catalyst, are not always compatible with the appropriate biological screening.

B. Lipids and Sugars

The CuAAC reaction has also been successfully adopted to join lipids to proteins. Indeed, although many proteins which are present in the cell membrane undergo a single or a dual lipidation, synthetic lipidation of proteins still represents a formidable goal and fast and reliable methods to achieve this are still lacking. To prove the feasibility of this, phosphatidylethanolamine was converted in the corresponding azide derivative, which was successfully reacted with a terminal alkyne protein (e.g., 32) (Fig. 12).

The click-triazole formation has been also used to generate simplified synthetic mimics of bolaamphiphiles, polar lipids that allow archaebacteria to live under extreme conditions of heat and pH. They have membrane-stabilizing properties that could be particularly useful for drug delivery applications but they are difficult to synthesize in large amounts. In order to add a terminal alkyne group, one palmitic acid chain was cleaved enzymatically from dipalmitoylphosphatidylcholine and replaced with an undecynoic acid chain. In order to generate stable homodimers, two molecules of the alkyne derivative were reacted with various bis-azides in the presence of copper sulfate/sodium ascorbate forming three compounds. These were unable to form vesicles but one compound (33) (Fig. 12) displayed vesicle disrupting properties.

Thus, the synthesis of lipoprotein and lipid analogues via click chemistry should allow an increased number of tools to understand lipid behavior in biological settings, including the role of compartmentalization of specific lipids in membranes (e.g., lipid rafts).

**Figure 12.** Lipidic structures containing the triazole ring.
Click chemistry has also had a major impact in carbohydrate chemistry. Indeed, examples exist where one or more sugars have been linked to a central core (drug, peptide, etc.), or where functional groups have been attached to sugars themselves. For example, the cyclic decapeptide tyrocidine (34) (Fig. 13), which has been demonstrated to have antibiotic properties targeting the lipid bilayer of bacteria, has been coupled to different sugar moieties in order to improve its safety profile. Two hundred forty-seven new glycosylated cyclic peptides were generated through the insertion of mono-, di- and tri-saccharides. These compounds were then submitted to biological screening in order to calculate the minimal inhibitory concentration and the minimal haemolytic concentration (the most important side-effect of tyrocidine). Two of the synthesized triazole-grafted glycopeptides (e.g., 35) (Fig. 13) showed a sixfold better therapeutic index compared to tyrocidine.56

CuAAC reactions have been used to link multiple sugars upon a central aromatic scaffold57 and a similar strategy, although with different scaffolds and sugars, has been used to synthesize compounds with antitumoral potential. For example, taking advantage of preliminary data that showed that β-D-glucosamine hexamer (36) showed mild antitumoral potential,58 a C3 symmetric (1-6)-N-acetyl-β-D-glucosamine octadecasaccharide (37) was prepared via triazole-linkage in order to exploit the concept of multivalency (Fig. 14). As hoped, the trivalent octadecasaccharide was shown to have better tumor growth inhibition properties compared to the monovalent controls.59

CuAAC has also been used to insert different molecules on an azide-sugar linked to vancomycin. In detail, the authors first coupled sugars to the antibiotic via enzymatic glycorandomization and then used CuAAC to generate a library of potential antimicrobial compounds.47,60 It is evident that coupling powerful synthetic strategies in this manner allows the generation of huge libraries in a relatively short period of time.

Amino acid–saccharide conjugates have also been synthesized via CuAAC chemistry using readily available carbohydrate and amino acid derived azides and alkynes as building blocks. The basic idea for this work was the finding that synthetic oligosaccharides covalently attached to proteins facilitate the development of vaccines against a series of pathogenic bacteria (e.g., 38) (Fig. 15).61 In an even more spectacular way, Danishefsky and co-workers combined three tumor-associated
carbohydrate antigens on a single molecule polypeptide scaffold (39) taking advantages of multiple click triazole formations (Fig. 15).62

Finally, the linkage of sugars to poorly soluble drugs via CuAAC might be exploited to improve the pharmacokinetic profile. This has been shown to be possible for ferrocene derivatives that have antimalarial activity. The authors increased the solubility of the desired ferrocene derivative by coupling it to sugars using click chemistry (e.g., 40) (Fig. 15). Although no biological data is available, it was demonstrated that these compounds maintain similar electrochemical behavior compared to ferrocene (Fig. 15).63 In another report, sugars have also been used to generate more water-soluble carbonic anhydrase inhibitors by “clicking” them with the classical recognition fragment able to bind to the active site of carbonic anhydrases: the aromatic sulphonamide (e.g., 41) (Fig. 15). In this case, biological tests demonstrated that some of the compounds retained their biological activity while being more water-soluble.64

C. Bioconjugation

An exciting extension of using triazole as a linker between two chemical entities is the use of CuAAC to immobilize small molecules onto macromolecular structures. One of the first examples appeared in 2003, when a fluorescent dye was attached to cowpea mosaic virus. At first, the capsid was functionalized by inserting terminal azides or alkynes and subsequently, an azide- or alkyne-functionalized fluorescent probe was successfully grafted on the surface of the virus via triazole formation.65 Possible applications of this are endless. For example, the application of CuAAC to generate fluorogenic compounds has been extensively evaluated, since it would be a powerful method to track biomolecules in the cell. Indeed, among the most investigated strategies is the use of...
coumarin as the fluorescent probe where the fluorescent capacity of coumarins with an electron-withdrawing group in position 3 or 7 has been exploited. Indeed, fluorescent DNA probes, an indispensable tool in molecular biology, can be generated in this manner. Yet, it is also possible to generate fluorogenic probes by reacting non-fluorescent 3-azide coumarins with alkynes (Fig. 16), thereby generating in situ probes. Indeed, when this was attempted, further evidence of the amenability of CuAAC to fast parallel combinatorial approaches was provided, as the physical properties (fluorescence) of the products could be monitored directly in the reaction chamber (a 96-well microtiter plate; an approach described by the Authors as “click and probe”).

One of the great challenges of biology is understanding the function of proteins in their natural setting, including their regulation. In part this need is answered by traditional proteomic approaches (for example, 2D electrophoresis followed by mass spectrometry). Yet, these techniques are mainly
designed to compare protein levels, and therefore the activity of these proteins can only be inferred. In response to these limitations, activity-based protein profiling (ABPP) techniques are beginning to be established and fine-tuned. This strategy would allow the visualization of proteins expressed at low levels, and the information would be an indication of activity more than of abundance. In general, this strategy employs site-directed probes that bind, in a specific and covalent manner, to the active sites of enzymes. These site-directed probe are usually composed of two different elements: the molecule that brings selectivity to the binding and the label that can then be visualized (e.g., fluorescent probe, antibody-recognizable tag, etc.). These two components can be pre-assembled and added to cell extracts, and, indeed, this has been done to profile a number of enzymes. A CuAAC-based approach has been used to identify the target of the cytotoxic compound FR182877. In brief, azide-derivatized FR182877 was reacted with alkyne-derivatized fluorescent or biotin probes. The products were then incubated with tissues, and the probes were used to purify/detect the bound target (i.e., carboxylesterase-1).

The tag is usually a large molecule and does not pass cell membranes easily, preventing this strategy from being employed in vivo. To circumvent this problem, a few strategies have been engineered where the site-directed molecule is first introduced in cells, and this is reacted afterwards with the label in vitro. This latter step has been optimized with click chemistry. In brief, azide-derivatized probes are incubated with intact cells. This is followed by cell lysis and the proteins obtained are then reacted in vitro with an alkyne-tagged probe in the presence of copper to allow the detection of the complex (Fig. 17). This technique has been successfully used, for example, to compare the activity of various enzymes in breast cancer cells.

![Figure 16. Fluorogenic probe generated via CuAAC.](image)

![Figure 17. Schematic representation of the ABPP technique using the CuAAC reaction.](image)
Liposomes used to deliver drugs in a specific manner may also benefit from click chemistry. The success of targeted liposomes relies on the coupling of a specific ligand on the surface of the liposome to give cell-selectivity. The click reaction has been used to attach an unprotected α-D-mannosyl derivative or an azido modified lysine to the surface of a liposome. Even though these examples are just a proof of principle, they pave the way to more specific applications with biologically relevant ligands. These would then be used to specifically target the contents of liposomes to cells expressing the receptor for the “clicked” ligand. On the other hand, it should be stressed that the presence of copper in liposomes which contain unsaturated lipids appears to be troublesome, since copper can oxidize lipids, altering the integrity of the liposome. It is therefore evident that other click reactions should be devised for these applications.

D. Immobilization of Ligands

The CuAAC reaction has also proven to be useful for the immobilization of ligands to solid supports, in particular during the generation of microarrays. For example, chemoselective ligation linking single strand DNA to a gold-coated platform was achieved using copper (I) in the presence of a triazolylamine ligand. This ligand also prevented the production of potentially DNA damaging oxygen radicals via the redox chemistry of copper (I). In another paper, ligand immobilization was achieved using a tandem Diels–Alder and azide–alkyne cycloaddition. For example, it was possible to conjugate a variety of compounds (biotin, lactose, thrombomodulin) onto PEG functionalized glass slides. Click chemistry has also been applied to the generation of carbohydrate microarrays. This was achieved by reacting an aliphatic azide with the commercially available unsialylated disaccharide N-acetylacetosamine. It has been shown that these microarrays can then be incubated with enzymes of interest to identify new inhibitors with nanomolar activities. These last reports impinge heavily on the future of both nucleic acids and protein microarray development for disparate applications such as basic research, disease diagnosis and drug discovery.

Another application of CuAAC-mediated immobilization is chromatography such as affinity chromatography, linking a ligand to a support via click chemistry. Indeed, it is possible to envisage that azide- or alkyne-resins could be bought by scientists and the reaction with the desired specific ligand be performed in-house. Furthermore, CuAAC has been used to generate glycol-silica supports for affinity chromatography applications in order to overcome the strong electrostatic interactions between silica and proteins which can alter the secondary structure of the protein and diminish its catalytic activity.

E. Click Chemistry and Multicomponent Reactions

The potential of click reactions can be further amplified by combining it with multicomponent reactions. Multicomponent reactions (MCRs) are reactions where three or more substrates combine in one step to give a product that contains essential parts of all of them. The combination of a classical multicomponent reaction with a classical organic transformation has been shown by many to be a powerful strategy to yield complex structures in few synthetic steps. The idea of using a MCR followed by a Huisgen [3 + 2] copper catalyzed reaction was first presented by Barbas and co-workers. In this paper, a multicomponent reaction between a phosphorane, an aryl aldehyde and a spirolactone, using an L-proline catalyst, gave various dispirolactones through a domino Wittig/Knoevenagel/Diels–Alder reaction. Dispirolactones have been shown to possess antioxidant and free radical scavenger activities. These compounds were then reacted with benzyl azide in the presence of copper sulfate/metallic copper to yield new spirotrione-1,2,3-triazoles, increasing the chemical complexity of these compounds (e.g., 42) (Fig. 18). The combined organo-catalysis and CuAAC approach (termed by the authors “organo-click”), would appear promising for the generation of diverse chemical libraries. In another paper, the Ugi reaction was coupled with an
intramolecular alkyne azide cycloaddition, giving a series of triazolobenzodiazepines and dihydrotiazolo[1,5-a]pyrazinones in high yield (e.g., 43) (Fig. 18). Although in this report there was no biological data, it is clear that this approach can be used to generate libraries for high throughput screening. In another example, a library of triazolyl-dihydropyrimidone analogues was synthesized under microwave irradiation, combining a Biginelli reaction with CuAAC (e.g., 44) (Fig. 18). Dihydropyrimidones are privileged structures endowed with a plethora of biological activities and the application of the click reaction allowed the straightforward generation of analogues which might maintain or improve their biological activities. Unfortunately, no biological data have been reported to date.

A new click multicomponent reaction was recently published where an acetylated Baylis–Hillman product was reacted with an alkyne and sodium azide in different solvents (water, ethanol, PEG 400) to give triazoles functionalized on the N-triazole ring (Fig. 18). Although the combination of click chemistry and MCR is powerful per se, it also represents formal proof that fast, efficient reactions can be combined to generate efficiently vast chemical libraries.

F. Macrocycles

Macrocycles often display remarkable biological activities and they can be seen as privileged structures in medicinal chemistry. Several factors are responsible for these attractive properties. For example, macrocycles, unlike their linear counterparts, are able to adopt fewer energetically favorable conformations, thus reducing the entropic penalty related to the binding to the target and increasing their biological activity.

The strong exoergic azide-alkyne reaction (30–35 kcal/mol) has been utilized to afford diverse macrocycles. Indeed, it is possible to predict that the gain in enthalpy counteracts the loss in entropy in this type of reaction; furthermore, copper could act as a handle, favoring the approaching of the two halves and giving the desired macrocycle product.

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The first example of this strategy was carried out in solid phase to cyclize short peptides and the result obtained was spectacular. Strikingly, the products obtained were solely cyclodimers, with two triazoles joining the two ends of the linear peptides (76 (45) and 126 (46) membered rings) (Fig. 19).87 To explain this interesting behavior Finn and co-workers proposed that a coordination of two terminal alkynes prior to the cycloaddition is responsible for the preferential formation of the dimeric product.

The difficulty in obtaining monomeric macrocyclized products has also been shown in solution, during the synthesis of more stable β-turn mimetics.88 Starting from the linear precursor, CuAAC was used to generate a 14-membered constrained dipeptide macrocycle but a mixture of both the monomeric (47) and dimeric macrocycles (48) was obtained in very poor yield with a strong predominance for the dimeric form (Fig. 19).

Yet, monomers can be also produced, as has been shown with the cyclopeptide pro-tyr-pro-val which was obtained in excellent yield through a CuAAC protocol (49) (Fig. 20). This peptide, first isolated in 1983 from the bacteria Lactobacillus helveticus, has shown to be a potent tyrosinase inhibitor but its total synthesis has not yet been reported, due to the difficulty in cyclizing the linear precursor. By capitalizing on the bioisosteric potential of triazole as an amide surrogate, the authors were able to prepare the desired cyclic tetrapeptide with an excellent yield of 70%.89,90 It is interesting to note that no formation of dimeric products was reported. Similarly, the ability of the 1,4-disubstituted triazole ring to mimic Z-amide bonds led to a macrocyclization reaction in order to synthesize a series of pseudohexapeptides which might be useful both in biology and in material sciences (e.g., 50) (Fig. 20).91

Figure 19. Macrocycles synthesized via the CuAAC reaction.
Libraries of macrocycle derivatives exploiting the CuAAC reaction to perform the cyclization step have been prepared by two different groups. In the first instance, a series of different diastereoisomeric macrocycles was successfully synthesized via a three subunit-system (e.g., 51): the central scaffold, and two interchangeable units. In the second report, the macrocycles were synthesized in a one pot reaction, combining a three component reaction, involving an α-isocyanoacetamide, an aldehyde and an amine, appropriately functionalized with an azide or an alkyne, and the 1,3-dipolar cycloaddition (e.g., 52). It is quite remarkable that in both of these examples, a heterocyclic ring was present (a furan and an oxazole, respectively), which rigidifies the molecules rendering them more susceptible to the intramolecular [3 + 2] cycloaddition (Fig. 20).

Macrocyclic carbohydrate-amino acid hybrids have also been prepared by macrocyclization via CuAAC. These structures have been postulated to act as potential artificial receptors. It could be thought that the presence of a triazole ring affords a more rigid macrocycle with a larger cavity for more efficient guest molecule binding. To test this hypothesis, C2-symmetric carbohydrate-amino

**Figure 20.** Macrocycles synthesized via the CuAAC reaction.
acid hybrid macrocycles containing two triazole rings were synthesized (e.g., 53) (Fig. 20). Molecular modeling calculations of these molecules showed that the low energy conformer generated by CuAAC displays a central cavity in contrast to the collapsed structure of the control, non-triazole containing structure.94

It appears that the intramolecular CuAAC reaction allows the generation of highly strained macrocycles. Indeed, the synthesis of 12- to 17-membered "triazolophanes", which could be developed in peptidomimetics, has been reported (e.g., 54) (Fig. 20). Strikingly, one of the reactions attempted, albeit catalyzed by copper, led to the selective formation of 1,5-regioisomer (54a) (Fig. 20). This unique observation can be explained by the fact the 1,5-conformation presented a lower molecular strain compared to the 1,4-regioisomer.95

G. Click Chemistry In Situ

The conventional approach used by medicinal chemists in drug discovery consists of the synthesis of a collection of compounds and subsequent biological screening. The procedure would be shortened and optimized if the biological target could actively choose its best ligand. Indeed, the target would act as the ideal template to generate the perfect hit. In the specific context of this review, it is therefore possible to envisage that, at room temperature, an enzyme could bring azide and alkyne containing molecules in close proximity so as to overcome the high energetic barrier allowing the Huisgen reaction to occur. In this manner, only compounds which fit correctly into the active site of the enzyme should react to give new potent ligands of the enzyme (Fig. 21).96–98 In this case, the active site of the enzyme would act as the reaction chamber. This strategy was used to identify extremely potent inhibitors of acetylcholinesterase (AChE), the key enzyme that hydrolyses acetylcholine at the synaptic cleft and a pharmacological target for many clinically relevant conditions, including Alzheimer’s disease. A set of tacrine, phenanthridinium and, other analogues derivatized with an azide or a terminal alkyne were incubated with the enzyme in conditions similar to those presented endogenously (Fig. 21). Of all possible sets of reactions (>75 combinations), only 6 combinations took place, affording small amounts of the 1,5-disubstituted triazoles. These compounds have been shown to be the most potent non-covalent inhibitors of Ache discovered to date with dissociation constants in the fentomolar range (KdS 33–100 fM). This compound revealed is the most potent

Figure 21. Click-chemistry in situ. On the left side, known ligands of acetylcholinesterases modified with either an alkyne or an azide group await in the active site of the enzyme for azide-or alkyne-containing partners that can fit the enzyme. No copper is required for the reaction to occur, as vicinity dictates the efficiency of the reaction. On the right side, the 1,5-disubstituted triazole formed.
inhibitor of acetylcholinesterase discovered to date, with an impressive $K_d$ of 77 femtomolar. It is interesting to note that the 1,4 isomer was not formed in situ. As expected, when this latter compound was synthesized by traditional methods, it was found to be a weaker inhibitor compared to the 1,5-disubstituted triazole, giving an explanation as to why it was not formed in situ.\textsuperscript{97,98} It should be noted that this approach does not involve the presence of a catalyst (e.g., copper), although it is commonly defined “in situ click chemistry”.

After this pioneering report, it has been shown that this strategy can also be used with other enzymes. For example, an experiment was set up using an HIV-1 protease and an alkyne and an azide which, when linked by a CuAAC-formed 1,4-disubstituted triazole, generate a known inhibitor of this enzyme. Indeed, the inhibitor formed spontaneously in the active site of the enzyme. In this case, the 1,4-disubstituted triazole was formed in the presence of the enzyme in a predominant manner with a

\begin{figure}
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\caption{Selected examples synthesized using the CuAAC reaction with possible applications.}
\end{figure}

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ratio of 18:1 compared to the 1,5-disubstituted triazole. When different alkynes were introduced into the same mixture as a confounding element, the selective formation of the known inhibitor was observed, demonstrating that the enzyme can indeed guide specificity.\textsuperscript{8a} The applicability of this technique was also demonstrated for carbonic anhydrase inhibitors,\textsuperscript{99} strongly suggesting that this powerful technique is applicable to many drug targets. Indeed, although only enzymes inhibitors have been synthesized to date, there is no reason to assume that receptor ligands could not also be identified in this manner.\textsuperscript{100}

Image 23. Selected examples synthesized using the CuAAC reaction with possible applications.
In conclusion, we believe that the examples listed in this review leave no doubt that click chemistry is, and will be, an important aid to the medicinal chemist. Although this review does not want to be exhaustive, but just describe selected examples of the applications of CuAAC in medicinal chemistry, Figures 22–24 show an array of compounds synthesized but not discussed in this work.

Although variations of the reaction exist, and probably better reactions will be described and applied in the future, the general principle of efficiency, versatility and selectivity will be maintained if not improved. For example, there is the need to synthesize NH-1,2,3-triazole derivatives using the CuAAC approach, to expand the versatility of this reaction, as click chemistry with sodium azide is

4. FUTURE PROSPECTS

In conclusion, we believe that the examples listed in this review leave no doubt that click chemistry is, and will be, an important aid to the medicinal chemist. Although this review does not want to be exhaustive, but just describe selected examples of the applications of CuAAC in medicinal chemistry, Figures 22–24 show an array of compounds synthesized but not discussed in this work.

Although variations of the reaction exist, and probably better reactions will be described and applied in the future, the general principle of efficiency, versatility and selectivity will be maintained if not improved. For example, there is the need to synthesize NH-1,2,3-triazole derivatives using the CuAAC approach, to expand the versatility of this reaction, as click chemistry with sodium azide is
not optimized. Recently, a significant step forward was made when the use of azidomethylpivalate was reported. After the CuAAC reaction, the protecting group can be easily cleaved using mild basic conditions to give the desired NH-1,2,3-triazole derivative which is amenable of further derivatization.\textsuperscript{101} β-Tosylethylazide can also react with alkynes under the copper catalyzed conditions to give the desired protected triazole. Deprotection has been achieved easily using potassium \textit{tert}-butoxide at $-78^\circ$C. It appears interesting that in this instance, internal alkynes can also react with copper (acetylene dicarboxylic acid and its dimethyl ester). As without copper no cycloaddition product can be detected and these alkynes cannot afford copper acetylenes, how this reaction occurs remains a mystery to be solved.\textsuperscript{102}

Remarkable is also the recent report of the use of CuAAC to generate 1,4,5-trisubstituted-1,2,3-triazole. Indeed, the presence of the $N,N,N'$-trimethylethlenediamine as the ligand gives trisubstituted triazoles, where two alkynes react with one azide. This might therefore, also be classified as an AAB multicomponent reaction.\textsuperscript{103}

The synthesis of bis-triazole using the typical Sharpless condition will also further expand the aim of this reaction both in medicinal chemistry and material science. In a very recent paper the conditions to build two triazole linkages in one pot were reported. The success of this operation lies in the full compatibility between the copper (I) catalysts and silver (I) salts where the latter catalyze the deprotection of trimethylsilyl alkynes in a hydroalcoholic medium.\textsuperscript{18}

As mentioned, a key question is the bioisosteric potential of the triazole ring and, once this is more fully evaluated, this reaction will enter the routine SAR of the medicinal chemist. Yet, it would be limiting to state that these are the main advantages of click chemistry. Indeed, the possibility to generate arrayed libraries, together with the possibility to employ click chemistry coupled with multicomponent reactions or CuAAC in solid phase, will make this reaction important in the generation of chemical libraries prior to screening. In brief, click chemistry has the potential, if appropriately exploited, to shorten and render more efficient lead identification and lead optimization procedures in medicinal chemistry. In this context, it is easy to predict the usefulness of this reaction in fragment-based ligand design.\textsuperscript{104} Triazole peptidomimetic fragments have been generated and these could act as building blocks to be used in the fragment-based approach to drug discovery, thereby rendering this technique feasible.\textsuperscript{105} The possibility of creating protein, DNA or carbohydrate microarrays or equivalents for nanotechnological applications via click chemistry is by no means a lesser achievement, as are other applications in the material sciences.

Finally, it is crucial to highlight that click chemistry has been shown to be able to help bridge the gap between chemistry and biology, becoming a true interdisciplinary reaction. Indeed, click chemistry can directly link chemistry to biology (such as in the ABPP assay) and can use biology for creating tailored syntheses.

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