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Review Article

CLICK CHEMISTRY AS A POWERFOOL TOOL IN PHARMACEUTICAL APPLICATIONS

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ABSTRACT

The traditional process of drug discovery based on natural secondary metabolites has often been slow, costly, and labor-intensive. Even with the advent of combinatorial chemistry and high-throughput screening in the past two decades, the generation of leads is dependent on the reliability of the individual reactions to construct the new molecular framework. Click chemistry is a newer approach to the synthesis of drug-like molecules that can accelerate the drug discovery process by utilizing a few practical and reliable reactions. Click chemistry refers to a group of reactions that are fast, simple to use, easy to purify, versatile, regions pecific, and give high product yields. Of the reactions comprising the click universe, the Huisgen 1,3-dipolar cycloaddition of alkynes has emerged as the frontrunner. Click chemical reactions have wide applications in many areas of pharmaceutical sciences such as synthesis of lead discovery libraries, radiolabelling, drug delivery systems and bioconjugationetc. In this manuscript, an in-depth look will be taken at some of its applications pertaining to the field of pharmaceutical sciences and assess if, also in this chemistry domain, "everything is as easy as a click".

Keywords: Click chemistry, Bioconjugation, Cycloaddition, Radiolabelling.

INTRODUCTION

In the field of pharmaceutical science, researchers are constantly seeking for new molecules and constructs that exhibit specific properties. The vibrant dynamics of the pharmaceutical industry has lead to many novel technologies and innovative ideas for development of new chemical entities. Click chemistry is a modular approach that uses only the most practical and reliable chemical transformations. Click Chemistry describe reactions that are high yielding, wide in scope, create only byproducts that can be removed without chromatography, stereospecific, simple to perform, and can be conducted in easily removable or benign solvents. This concept was developed in parallel with the interest within the pharmaceutical, materials, and other industries in capabilities for generating large libraries of compounds for screening in discovery research. Its applications are increasingly found in all aspects of drug discovery, ranging from lead finding through combinatorial chemistry and target-templated in situ chemistry, to proteomics and DNA research, using bioconjugation reactions.[1]

Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC)

A well-known example is Cu (I)-mediated alkyne-azide cycloaddition, which has become known as the 'click reaction'. The click reactionproceeds via a Cu(I) intermediate (CuSO4) (Fig.1). Itsucceeds over a broad temperature range, is insensitive to aqueous conditions and a pH range over 4to 12, and tolerates a broad range of functional groups. Pure products can be isolated by simplefiltration or extraction without the need for chromatography or recrystallization.[2]

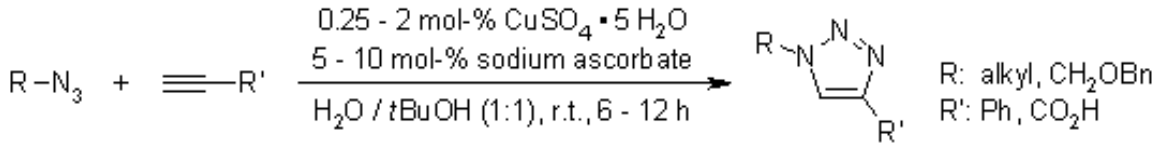


Fig. 1: Schematic representation of Cu (I)-mediated alkyne-azidecycloaddition (''click reaction'')

The Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) first described in 2001, has been established as a powerful coupling technology for the conjugation of biomolecules.[3-5] CuAAC reaction is the method of choice for DNA click chemistry due to its remarkable efficiency and it has been used to label oligonucleotides with fluorescent dyes, sugars, peptides and other reporter groups, to cyclise DNA, to synthesis DNA catenanes, to join oligonucleotides to peptide nucleic acids, and to produce analogues of DNA with modified nucleobases and backbones.[6]Copper and ruthenium are the commonly used catalysts in click reactions.

Fluorogenic CuAAC reactions have emerged as apowerful tool for bioconjugation, organic synthesis anddrug discovery.[7]A suitable example of the application ofclick chemistry in biomedicine and diagnostics describesradiolabeled cyclic peptides and their use in imaging, such as Positron Emitting Tomography or Single PhotonEmission Computed Tomagraphy.[8] Click-functionalizedquantum dots coated with a new class of multifunctional multidentate polymer ligands provided a biologicalinterface necessary for living-organism, for example,virus labeling and imaging.[9] "Stepwise and double click" chemistry concept combined with selective hybridizationrepresents a flexible tool to generate DNA nanostructureswhich are used for various purposes in DNA diagnostics,delivery, and material science applications. Branched DNA was synthesized from tripropargylated oligonucleotides by the Huisgen-Meldal-Sharpless cycloaddition using" stepwise and double click" chemistry.[10]

APPLICATIONS OF CLICK CHEMISTRY

Of the click chemistry reactions, the CuAAC reaction currently has the greatest potential for commercialapplication in (i) materials science and (ii) life sciences.

Specific applications in material science include the making of antibacterial, anti-immunogenic coatings for medical implants or metal coatings for semiconductors.

In the life sciences category, bioconjugation is oneof the most commonly cited uses of click chemistry.

Click in Bioconjugation

Bioconjugation describes a technique in which asynthetic label (e.g. fluorophores, ligands, chelates, orradioisotopes) is covalently linked to a biomolecules (e.g. proteins and nucleic acids). Although bioconjugation is applicable to the *in vivo* labeling of biomolecules, only a handful of reactions are actually useful.[1, 11] The possibility of applying click chemistry in bioconjugation was first demonstrated by Tornoe et al. for the preparation of peptidotriazoles via solid-state synthesis.[12] Their goal was to develop new, more efficient synthetic methods to prepare various [1,2,3]-triazole pharmacophores for potential biologic targets. This initial report makes possible the introduction of various novel functional and reporter groups into biomolecules, such as peptides and proteins, [13] for DNA labeling and modification, [14-16] and for cell-surface labeling.[17] Most bioconjugation reactions, such as isothiocyanateamine, thiol-maleimide, and amine-carboxylic acid couplings, [18-21] cannot be used for labeling *in vivo*because of competing nucleophiles on proteins, nucleic acids, and other biopolymers. Labeling of biomolecules in living systems, using condensation reactions between ketones or aldehydes, and hydrazides or aminoxy derivatives, is not feasible. At the optimum pH of 5–6, such linkages are reversible, and ketones or aldehyde functionalities are present inside of cells.18 Click chemistry overcomes these obstacles by being bioorthogonal and by proceeding irreversibly in water at neutral pH and biocompatible temperatures (25–37°C) without any cytotoxic reagents or byproducts. Click chemistry continues to attract attention for the labeling of proteins and live organisms. In one study, conducted by Wang et al., fluorescein dye derivatives were attached to both azide-functionalized and alkyne-functionalized CPMV using click chemistry.[22] Not only were all of the reactions successful, but also their product yield was as high as 100%. Two years later, in 2005, they were able to attach three more hemicyanine dyes to CPMV using the same reaction.[23] Purification can be simply performed by dialysis or ultrafiltration.

PEGylation is an important application of bioconjugation and has become a widespread tool in pharmaceutical industries. The most commonly employed method for site-specific PEGylation relies on the use of Cu-catalyzed click chemistry. The site-specific PEGylation is achieved by incorporation of an azido-containing non natural amino acid, i.e., a homoazidoalanin into a recombinant protein that allowsfor site-specific conjugation using Cu-catalyzed click chemistry. The azide moiety serves as the attachment point for covalent modification of the protein with alkyne-PEG molecule via Cu-catalyzed click reaction. Attachment of the PEG molecule to the azideresidue is highly specific because of inherited selectivity ofclick chemistry. One major shortcoming of the Cu-catalyzed click reaction is the need for a highly toxic Cu(I) as well as Cu(II). Even in small amount, copper can damage proteins,in particular fluorescent proteins, like GFP. Therefore, in order for the click reaction to find in vivo applications, the copper catalyst must be completely removed. This may not always be an easy task, but a few research groups have demonstrated some success. As an example, in the click-PEG delivery system developed by Liu et al.[24], about 98% of the copper was effectively removed by incubation with ethylenediaminetetraacetic acid (EDTA), followed by dialysis (unpublished data). However, this approach can only be applied to large molecular weight structures. Another group, Veinot et al., has used oxide-capped metallic iron nanoparticles [25] as Cu sequester. After performing a click reaction with phenyl azide and benzyl acetylene, the crude product was incubated with the nanoparticles twice and filtered twice. Analysis revealedthat the copper concentration had been significantly reduced from 2,026 ppm to 4.6 ppm.

Click in Radio labelling

Radiolabeling has become a powerful tool in the pharmaceutical scientist's arsenal. By connecting a radionuclide to a drug of interest, one can track its in vivo distribution [26], what specific receptors it attaches to[27], its metabolic pathway[26], etc. In radiopharmaceutical chemistry where short lived radioisotopes are introduced into various different substance classes for in vivo imaging of biochemical processes, the expanding field of radioactive bioconjugation has become predominant. Labeled biomolecules such as peptides, proteins and oligonucleotides generated via bioconjugation of chelators for radiometal introduction as well as novel valuable secondary precursors for (18)F labeling have enriched the growing field of molecular imaging substantially. When introducing radioactive nuclides with a very short half-life into biomolecules, some of the typical criteria defined by click-chemistry are more crucial than others. Time is always the most important issue, whereas avoiding the formation of by-products that have to be removed without chromatography is of minor importance. The short-lived radionuclide ^{11}C for example has a physical half-life of only 20 min so that the labeling procedure cannot exceed 40-60 minutes (2-3 half-lifes). The short half-life demands rapid, efficient methods for the introduction of ^{11}C into biomolecules, because the specific activity (in MBq/μmol) is a function of time. The feasibility to apply click chemistry for the preparation of ^{11}C-labeled compounds was explored by Schirrmacher et al.[28]They reported a method to prepare a ^{11}C-labeled compound within 5–10 minutes under nontoxic aqueous conditions with the radiochemical yield of 60% at room temperature.

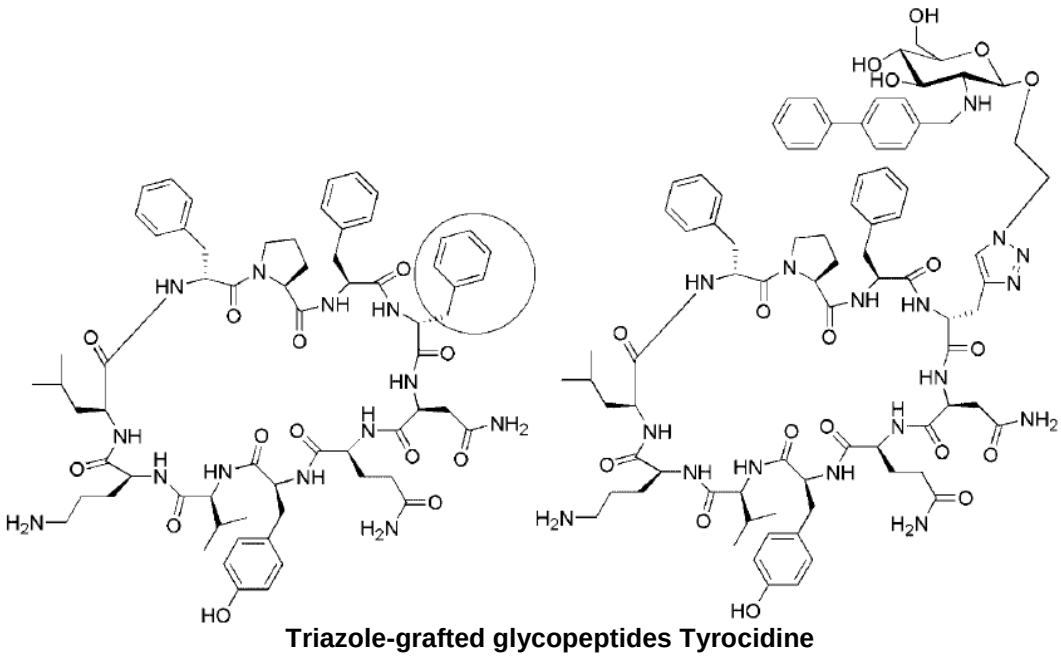
Click in DNA labelling

The attachment of labels onto DNA is of utmost importance in many areas of biomedical research and is valuable in the construction of DNA-based functional nanomaterials. The copper(I)-catalyzed Huisgencycloaddition of azides and alkynes (CuAAC) has recently been added to the repertoire of DNA labeling methods, thus allowing the virtually unlimited functionalization of both small synthetic oligonucleotides and large gene fragments with unprecedented efficiency. The CuAAC reaction yields the labeled polynucleotides in very high purity after a simple precipitation step. The reviewed technology is currently changing the way in which functionalized DNA strands are generated cost-efficiently in high quality for their application in molecular diagnostics systems and nanotechnological research.[29]The concept of "stepwise and double click" chemistry combined with selective hybridization represents a flexible tool to generate DNA nanostructures useful for various purposes in DNA diagnostics, delivery, and material science applications. In this context, branched DNA was synthesized from tripropargylated oligonucleotides by the Huisgen-Meldal-Sharplesscycloaddition using "stepwise and double click" chemistry. Dendronized oligonucleotides decorated with 7-tripropargylamine side chains carrying two terminal triple bonds were further functionalized with bis-azides to give derivatives with two terminal azido groups. Then, the branched side chains with two azido

groups or two triple bonds were combined with DNA-fragments providing the corresponding clickable function. Both concepts afforded branched (Y-shaped) three-armed DNA. Further, annealing of branched DNA with complementary oligonucleotides yielded supramolecular assemblies.[10]

Click in Carbohydrate chemistry

Carbohydrates make up 5% to 10% of plasma membrane mass, in the form of glycoconjugates, and mediate a variety of events, such as cell-cell recognition, metastasis, fertilization, and immunological response[30, 31]. Alterations to cell surface oligosaccharides have been linked to a number of different diseases, including cancer and tuberculosis [31]. Despite these pivotal biological functions, however, carbohydrates are rarely found in pharmaceutics. Their synthesis typically involves multiple reaction steps and many stereoisomers, making it challenging to even the most skilled chemists. Furthermore, theirmoderate affinity towards target receptors and enzymes and poor pharmacological properties make them seldom used astargeting moieties/lead compounds. 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 decapeptidetyrocidine, 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(Fig.2) showed a sixfold better therapeutic index compared to tyrocidine.[32]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.[33,34] It is evident that coupling powerful synthetic strategies in this manner allows the generation of huge libraries in a relatively short period of time.



Triazole-grafted glycopeptides Tyrocidine

Fig. 2: Tyrocidine and a triazole-containing glycosylated analogue

Click in Peptide chemistry

In 2002, the first application of the CuAAC reaction to form peptide derivatives was reported with the synthesis of peptidotriazoles and neoglycopeptide-linked-triazoles on solid support by Tornoeet al.[12] A wide variety of

azido groups were tested, affording compounds with crude purities ranging from 75% to 99% (Figure 3). They performed solid-phase syntheses of peptide-peptide conjugates by reacting a peptide-containing alkyne group with its counterpart peptide-containing azid group to yield peptidotriazoles (>95%).[35]The application of this reaction is not limited to conjugation between molecules (intermolecular coupling), but also within molecules (intramolecular coupling), thus the number of publications that have used this method have grown exponentially Similarly, Gogoi et al. developed a versatile method where peptide-linked terminal alkynes were allowed to react with nucleic acids containing an azide functionality and vice versa in both solid and solution (i.e., water) phases to obtain peptide-oligonucleotide conjugates.[36]This method proved to be far more efficient than the normally used (4 + 2) Diels-Alder cycloaddition, minimizing cross-reactions between the dienophile and other nucleophilic centers on the peptides.[37]The copper-catalyzed click reaction has been used to modify proteins with high selectivity under physiologic conditions. Deiters et al. demonstrated this novel approach by adding amino acids to the genetic code of *S. cerevisiae.*[38] The method involved incorporation of either azide- or alkyne-containing amino acids genetically inserted into proteins in response to the amber nonsense codon (TAG). The cycloaddition reaction was then performed with their counterpart alkyne or azide to study and manipulate cellular processes in eukaryotic cells. Staudinger ligation is also useful for the synthesis of large biomolecules. Most peptide syntheses require a terminal Cys residue at the active site and are hampered by low yields and inconvenient work-ups.[39]Nilsson et al. traversed those obatacles by developing a high yield Staudinger ligation method that does not require a Cys residue.[40-42]As shown in Fig.4, an amide bond was formed from a peptide-linked phosphinothiol at the C-terminus and a peptide-linked azide at the *N*-terminus. The end-product, an amidophosphonium salt, formed after the intramolecular rearrangement of the intermediate iminophosphorane with > 90% yield.

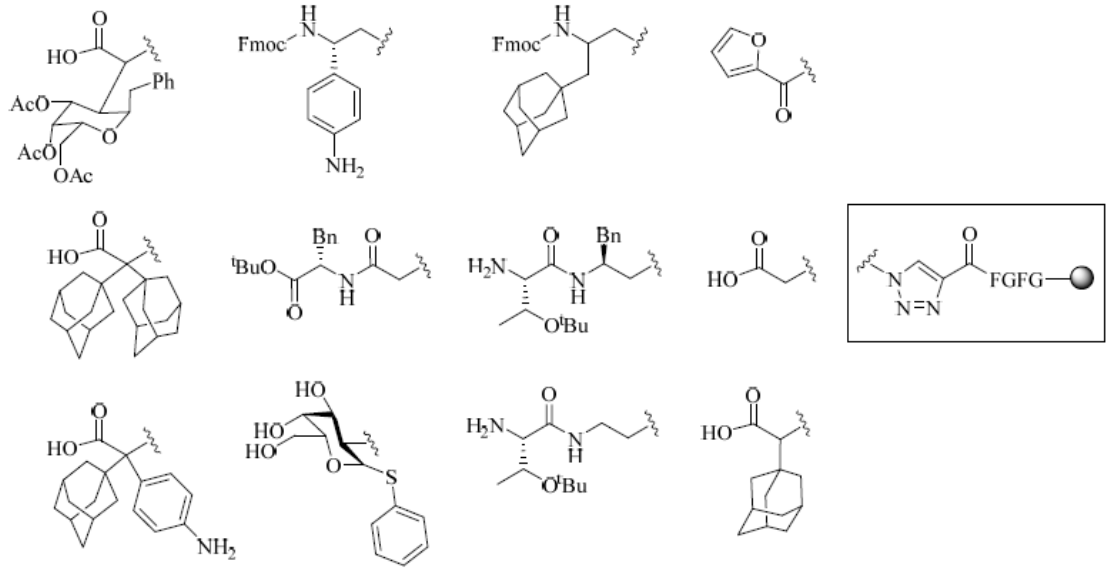


Fig. 3: Examples of resin-bound peptidotriazoles constructs synthesized via CuAAC

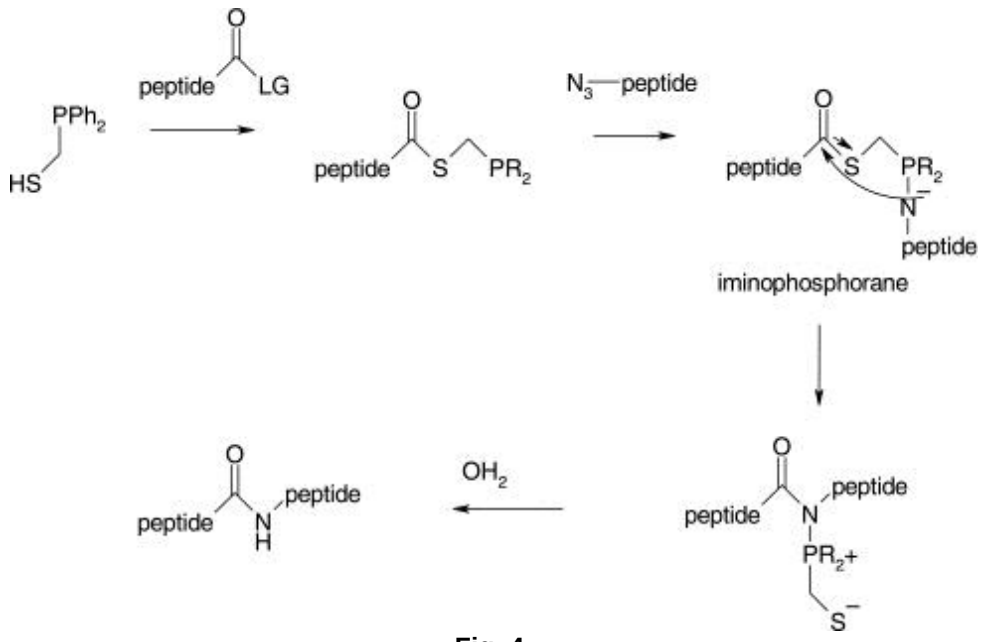


Fig. 4:

Click in Nanoscience

Click chemistry reactions were utilized as intermolecular cross-linking procedures to entrap peptide therapeutics and siRNA during the formation of the 3-D nanosponges. *In vivo* tumor growth delay studies in two cancer models with peptide targeted nanosponges showed a five times increased efficiency in comparison with traditional chemotherapy. Dendritic molecular transporter as cell penetrating units have been tested to deliver antibodies and peptides into cells.[43] Researchers have developed nanoparticles that are functionalized with highly-strained and highly-reactive cycloalkynes. These functionalized nanoparticles undergo spontaneous, reagent-free covalent reaction with metabolically incorporated azido-sugars on the cell surface, which is anticipated to promote nanoparticles internalization through endocytosis.[44] Numerous examples of click reactions have been reported for the preparation and functionalization of polymeric micelles and nanoparticles, liposomes and polymersomes, capsules, microspheres, metal and silica nanoparticles, carbon nanotubes and fullerenes, or bionanoparticles. Further, the impact of click chemistry in nanosized drug delivery systems was reported.[45]Nanoparticular delivery systems are the carriers ranging in size from 10 to 1000 nm. Over the past few decades, delivery systems such as quantum dots, magnetic nanoparticles, gold nanoparticles, micelles, and liposomes have all been extensively investigated for imaging and drug/gene delivery applications.[46,47] Due to the small size, they are able to penetrate through fenestrated vasculature, allowing efficient drug accumulation at target sites. Moreover, drug targeting by nanoparticuler delivery systems offers several important advantages: they reduce drug dose, minimize side effects, protect drugs against degradation, and enhance drug stability.[48] Researchers have demonstrated conjugation between azide-functionalized gold nanorods and an acetylenefunctionalized enzyme(trypsin) through click chemistry. Here, the click conjugated enzyme showed substantially improved specificity and activity compared to the same enzyme linked to the gold nanorod by conventional bioconjugation chemistries.[49]

CONCLUSION AND PERSPECTIVES

Despite the fact it debuted less than 15 years ago, click chemistry is quickly revolutionizing the way scientists view their research. Click chemistry is finding a number of applications in the areas of drug discovery, bio-conjugation, radiolabelling, material science, nanotechnology, supra-molecular chemistry, peptide/protein modifications chemistry, biomedicine and pharmaceutical science. Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of azides and terminalalkynes has emerged as the most popular click reaction by far. Research work is in progress in the area of click peptidomimetics which are prepared by the click coupling of alkynefunctionalized α-amino acid derivatives and α -azido acid compounds.Investigators are

exploring the possibility of building novel conformations such as β-turn, helical structure usingclick chemistry. The bioisosteric potential of the triazolering should be exploited further so that medicinal chemistscan use it in routine structure activity relationships.

Though the Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of azides and terminal alkynes has provides a major breakthrough in synthetic organic chemistry, there are a few important limitations that need to be considered. The most significant is it requires a copper catalyst. High level of copper in the body can lead to serious, even deadly, consequences. Another disadvantage involves Cu having variable oxidation states. Though this reaction has several limitations, it is still one of the most versatile and beneficial chemistry tools for pharmaceutical applications. We hope that it continues to grow in popularity and contribute to the major researches and advancements in the field of pharmaceutical science.

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