Azide – Alkyne Coupling: A Powerful Reaction for Bioconjugate Chemistry

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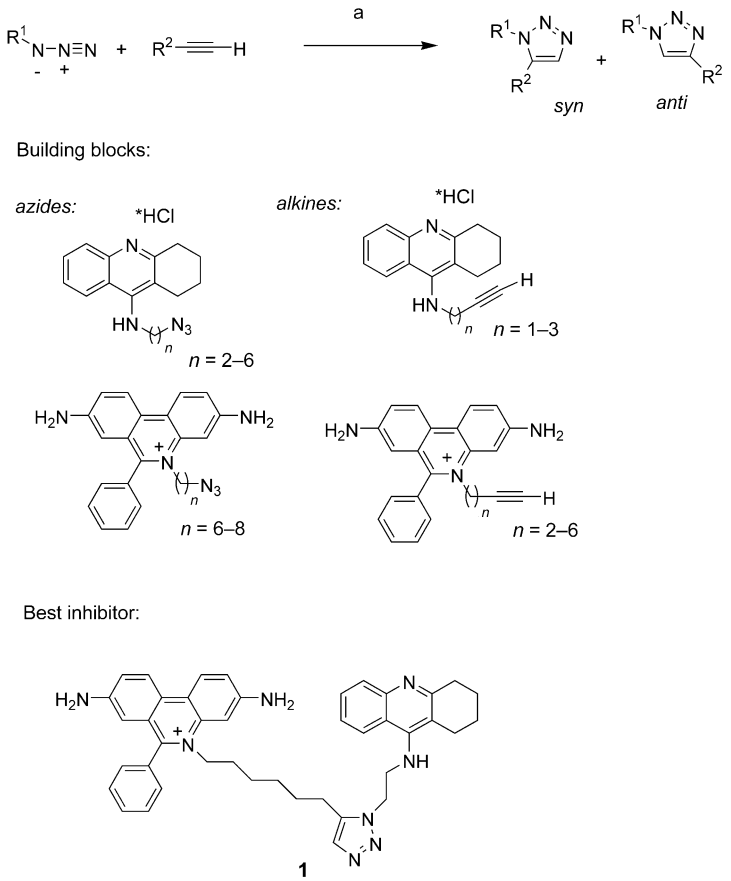
In their studies of biological systems, molecular biologists or chemists often encounter the challenge of covalently linking two molecular entities, for example, fusing two proteins together, linking a complex carbohydrate with a peptide, or attaching a small molecular probe (fluorescent dye, radical probe, affinity tag, etc.) onto a biopolymer. As biological systems are rich in structural complexity and diverse in functional reactivity, one has to find chemoselective ligation reactions that allow the coupling of two mutually and uniquely reactive functional groups, in most cases in an aqueous environment under physiological conditions. These uniquely reactive functional groups should be selective for each other and also tolerate a diverse array of other functionality, which renders the use of protecting groups unneccesary and, in the ideal case, allows application of the molecules in the complex environment of a living cell.

While several bioconjugation techniques are available for the in vitro preparation of bioconjugates exhibiting a limited set of functional groups,[1] truly chemoselective ligation reactions are rather limited. One reason for this problem is that most ligation reactions rely on the reaction of an electrophile with a nucleophile. As biological systems are rich in diverse electrophilic and nucleophilic sites, only a few functional groups are available that exhibit reactivity orthogonal to other functional groups existing within the system.[2] Recently, two reactions have been introduced that use the azide moiety as a functional group and offer two advantages: 1) the azide moiety is absent in almost all natural exisiting compounds and 2) despite a high intrinsic reactivity, azides allow selective ligation with a very limited set of reaction partners. Bertozzi et al. have redesigned the long-known Staudinger reaction between azides and phosphines so that the highly reactive aza-phosphor-ylide intermediate can undergo a follow-up reaction with cleverly positioned substituents on the phosphine to form a thermodynamically stable amide bond. The usefulness and the generality of this approach has been highlighted by the metabolic engineering of cell surfaces.[3]

Sharpless et al. realized that even higher functional group compatibility could be achieved if one could surpass the limitations imposed by electrophile – nucleophile reactions and turn instead to electrocylic reactions. The Huisgen 1,3-dipolar cycloaddition of azides and acetylenes to give 1,2,3-triazoles was identified as an interesting candidate for such an approach. This water-tolerant reaction is thermodynamically favorable by approximately 30 – 35 kcal mol^{-1}.

The groups of Sharpless and Finn applied the azide – alkyne coupling in the parallel synthesis of a highly active



*Scheme 1. Template-guided synthesis of the highly active AChE inhibitor **1** by azide-alkyne coupling. a) AchE (0.03 equiv), pH 7.4 buffer, RT, 49 parallel reactions.*

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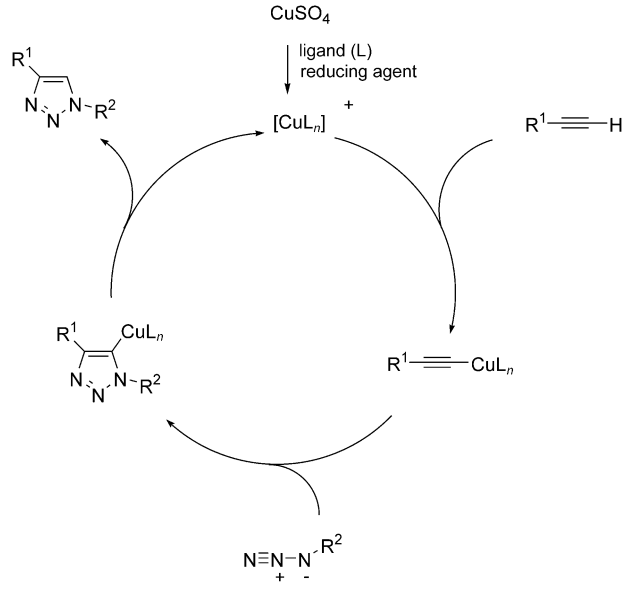
inhibitor of the enzyme acetylcholinesterase (AchE).[4] Unlike the conventional approach in which a diverse set of chemical compounds is first synthesized and subsequently screened against the biological target in question, the strategy chosen by Sharpless and Finn involves a biological target that should literally guide the inhibitor synthesis by serving as a template for the assembly of building blocks. Complementary pairs of a selection of site-specific inhibitors based on tacrine and phenanthridinium motifs decorated with alkyl azides and alkyl acetylenes of varying chain lengths were incubated in the presence of *Electrophorus* AChE at room temperature (Scheme 1). The rate of reaction under these conditions in the absence of enzyme is negligible, so detectable amounts of triazole products should form only when the azide and alkyne are brought together by the enzyme enforcing propinquity and proper alignment of the reactants. Of 49 reactions, only one combination led to a detectable amount of coupled product. This substance (**1**) turned out to be the most potent noncovalent AChE inhibitor known, with K_d values ranging from 77 fM (AChE from *Torpedo californica*) to 410 fM (mouse Ache).

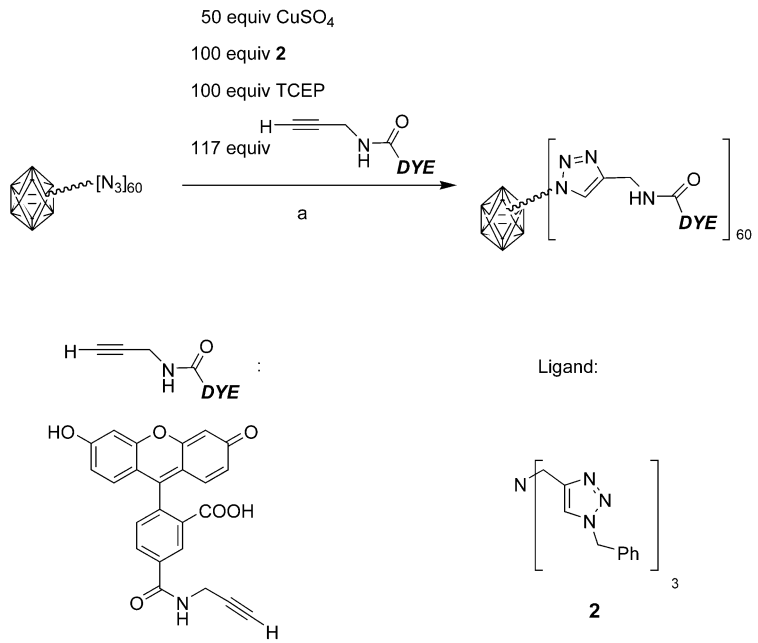
However, the low intrinsic reaction rate of the azide–alkyne coupling—key for the experiment described above—has to be dramatically increased to make this reaction attractive as a general bioconjugation technique. As increasing the reaction temperature is not an option because of the sensitivity of biological material to heat, a catalyst has to be identified that accelerates the reaction. The groups of Sharpless and Meldal have independently found out that catalytic amounts of Cu(I) salts not only dramatically increase the reaction rate, but also improve the regioselectivity to deliver exclusively the 1,4-disubstituted product (Scheme 2).[5] There are several Cu(I) sources available, but in situ reduction of inexpensive and air-stable $CuSO_4 \cdot 5H_2O$ by treatment with sodium ascorbate, phosphines (e.g. water-soluble tris(carboxethyl)phosphine (TCEP)), or a simple piece of copper wire turns out to be the method of choice. Addition of a tris(triazolyl)amine ligand (**2**) enhances the reaction rate greatly and inhibits oxidative alkyne coupling.[6]



Scheme 2. *Catalytic cycle of the Cu(I)-catalyzed azide–alkyne coupling.*

The Cu(I)-catalyzed reaction has been applied to solid-phase preparation of peptidotriazoles,[5b] fabrication of carbohydrate arrays,[7] and preparation of multivalent neoglycoconjugates.[8] In these reactions the Cu(I)-catalyzed azide–alkyne coupling proved to be compatible with the following functional groups: thioethers, carboxylic acids, esters, amides, ethers, thiols, alcohols, phenols, amines, guanidines, and carbamates.

An intriguing demonstration of the power of this chemoselective ligation reaction has been reported recently by Fokin, Sharpless, Finn et al.[6] They have succeeded in labeling all 60 identical protein units of the capsid of the cowpea mosaic virus (CPMV) with fluorescein (Scheme 3). The azide moiety was attached to either the NH_2 or the SH function of the virus capsid using conventional bioconjugation techniques.



Scheme 3. *Global labeling of CPMV virus with fluorescein dyes through Cu(I)-catalyzed azide–alkyne coupling (the shown shape of the virus is different from its real shape). a) Phosphate buffer/5% tBuOH, pH 8, 16 h, 4 °C.*

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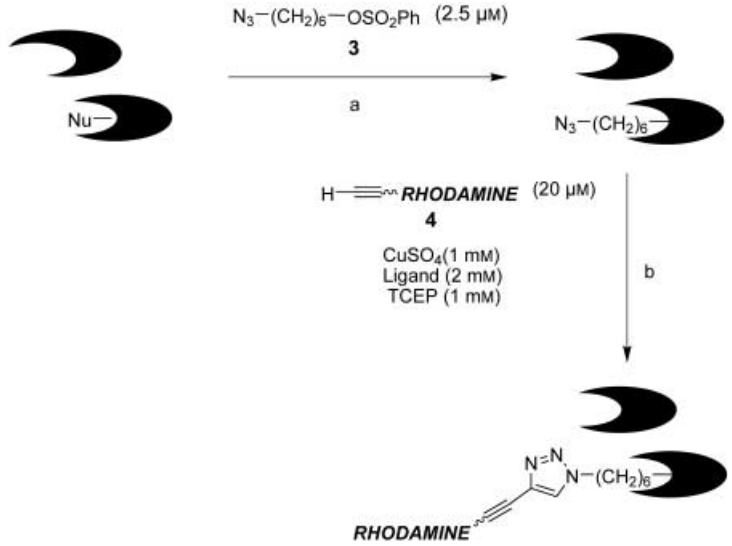

Scheme 4. *Activity-based protein profiling using the sterically inconspicuous azide probe* ***3****. a) Phosphate buffer, pH 8, 30 min, RT; b) phosphate buffer/5% tBuOH, pH 8, 1 h, RT.*

Recently, Cravatt et al. have applied the Cu(I)-catalyzed azide – alkyne [3+2] cycloaddition to the activity-based profiling of proteins (ABPP).[9] ABPP is a rather new postgenomic method in which affinity labels are used to profile proteins on the basis of their function in biological systems; this technique differs fundamentally from the conventional abundance-based methods of proteomics.[10] Whereas the commonly used reporter groups in ABPP (fluorophores, biotin, etc.) are quite bulky, which may inhibit cellular uptake and influence probe distribution within the cell, the newly designed azide derivative is sterically inconspicuous. Enzmyes containing a nucleophilic site were incubated with the azide-derivatized phenyl sulfonate ester **3** in phosphate buffer at pH 8 for 30 min (Scheme 4). The modified proteins were reacted with a Rhodamine-alkyne tag **4** for 1 h in the usual reaction cocktail (Scheme 4). The proteins were separated from excess reagents by centrifugation and analyzed by SDS-PAGE and in-gel fluorescence scanning. By using this approach Cravatt et al. were able to probe gluatathione *S*-transferase activity not only in cell lysates, but also in living cells and mouse tissue proteomes. Most exciting is their proof-of-concept experiment showing that ABPP with the azide probe **3** also works in vivo: Mice were injected with up to 20 mg kg^{-1} **3**, sacrificed after 1 h, and the homogenized heart tissued reacted with **4**. Fluorescence analysis delivered a clear signal indicating the presence of enoyl CoA hydratase, which was a target of the probe.

The examples described above have demonstrated that the recently introduced azide – alkyne coupling has become a powerful coupling method for chemoselective ligation. Although the reactivity of the participating reactive groups is almost perfectly orthogonal to those of the diverse functional groups in biological systems (only reduction of the azide group by free thiol groups has to be feared), this reaction still does not provide a final solution. The requirement of Cu(I) catalysis and some ancillary ligands to achieve reasonable reaction times imposes a severe limitation on in vivo use as such additional reagents have to be introduced into the biological system, where at least one—namely Cu—exhibits considerable cell toxicity. So far, this coupling reaction has not been performed in the environment of an intact cell, but only with cell lysates containing one reaction component. Nevertheless this conjugation strategy represents an important milestone on the way to the perfect ligation strategy.

Keywords: alkynes · azides · bioconjugates · bioorganic chemistry · chemical biology

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