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# The growing impact of click chemistry on drug discovery

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**Click chemistry is a modular approach that uses only the most practical and reliable chemical transformations. 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. The copper-(I)-catalyzed 1,2,3-triazole formation from azides and terminal acetylenes is a particularly powerful linking reaction, due to its high degree of dependability, complete specificity, and the bio-compatibility of the reactants. The triazole products are more than just passive linkers; they readily associate with biological targets, through hydrogen bonding and dipole interactions.**

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▼ The laborious process of lead discovery and optimization has, in recent years, been aided by combinatorial chemistry, to generate collections of test compounds for screening. However, due to the large number of compounds that are involved, combinatorial chemistry is even more dependent than 'traditional' synthetic chemistry on the reliability of the individual reactions used to construct the new network of chemical bonds. Click chemistry is a new approach to synthesis that greatly facilitates this process [1], making use of a few near-perfect chemical reactions for the synthesis and assembly of specially designed building blocks (Figure 1). These building blocks have a high built-in energy content that drives a spontaneous and irreversible linkage reaction with complementary sites in other blocks.

## The click chemistry approach

Click chemistry serves as a guiding principle in the quest for function: the search must be restricted to molecules that are easy to make. Focusing on lead discovery, this strategy provides a means for the rapid exploration of the chemical universe. For lead optimization, it enables rapid SAR profiling, through generation of analog libraries. Click chemistry does not replace existing methods for drug discovery, but rather, it complements and extends them. It works well in conjunction with structure-based design and combinatorial chemistry techniques, and, through the choice of appropriate building blocks, can provide derivatives or mimics of 'traditional' pharmacophores, drugs and natural products [1–3]. However, the real power of click chemistry lies in its ability to generate novel structures that might not necessarily resemble known pharmacophores.

Click chemistry is both enabled and constrained by its reliance on a few nearly perfect reactions, and this, naturally, raises concerns about limitations on its access to chemical diversity. A computational study by Guida *et al.* suggests that the pool of 'drug-like' compounds (<30 non-hydrogen atoms, <500 Daltons; only H, C, N, O, P, S, F, Cl and Br; likely to be stable in the presence of water and oxygen) is as large as $10^{63}$ [4]. Currently, only a few million compounds ($\approx 10^{6-7}$) that fulfill these criteria are known, implying that only an infinitesimal part of the potential medicinal chemistry universe has been explored, to date. This has staggering implications for drug discovery. First and foremost, the vast majority of molecules with useful properties remain to be discovered. Second, most useful new compounds are likely to be found in unconventional structure space.

Click chemistry-based searches are fast, because they avoid the regions of the '$10^{63}$-universe' that are difficult to access. They are wide-ranging, owing to the use of strongly driven, highly selective reactions of broad scope, allowing a much greater diversity of block structures to be used. Thus, click chemistry makes the interesting proposition that greater diversity can be achieved with fewer



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reactions, because it is not the number of reactions that is important, but the tolerance of those reactions to variations in the nature of their components.

### *Defining a click chemistry reaction*

Despite many successes, drug discovery approaches that are based on Nature's secondary metabolites, 'natural products,' are often hampered by slow and complex syntheses [2,5,6]. Through the use of only the most facile and selective chemical transformations, click chemistry simplifies compound synthesis, providing the means for faster lead discovery and optimization. A click reaction must be of wide scope, giving consistently high yields with a variety of starting materials. It must be easy to perform, be insensitive to oxygen or water, and use only readily available reagents. Reaction work-up and product isolation must be simple, without requiring chromatographic purification.

How does one identify and then use such near-perfect reactions to accelerate the discovery of better and cheaper drug substances? Nature's way in which she performs combinatorial chemistry serves as a source of inspiration: primary metabolism is highly modular – all proteins arise from 20 building blocks that are joined via reversible, heteroatom links (amides). Similarly, click chemistry uses carbon–heteroatom bond-forming connection chemistry. However, because we lack nature's ability to perfectly control reversible carbonyl chemistry, we focus exclusively on highly energetic, 'spring-loaded' reactants and pure kinetic-control of the outcome. Thus, reversible carbonyl chemistry and irreversible click chemistry lie near opposite extremes of the continuum that connects pure thermodynamic and pure kinetic control.

A focus on making carbon–heteroatom bonds must be accompanied by the use of pre-formed carbon–carbon bonds. The best, and most energetic, of these building blocks are olefins and acetylenes. Chemists have access to a plethora of such materials, ranging from naturally occurring terpenes to olefins from the petrochemical industry. They can easily be decorated via addition or oxidation reactions (Figure 1) [1].

### *The click chemistry universe*

A concerted research effort in our laboratories has yielded a set of extremely reliable processes for the synthesis of building blocks and compound libraries:

- Cycloaddition reactions, especially from the 1,3-dipolar family [7], but also hetero-Diels-Alder reactions [8,9].
- Nucleophilic ring-opening reactions, especially of strained heterocyclic electrophiles, such as epoxides, aziridines, cyclic sulfates, cyclic sulfamidates, aziridinium ions and episulfonium ions [1].
- Carbonyl chemistry of the non-aldol type (e.g. the formation of oxime ethers, hydrazones and aromatic heterocycles).
- Addition to carbon–carbon multiple bonds; particularly oxidation reactions, such as epoxidation [10], dihydroxylation [11], aziridination [12], and nitrosyl and sulfenyl halide additions [13], but also certain Michael addition reactions.

Over the past five years, we have observed that the very best click reaction classes proceed most rapidly and in highest yield, not in water or water–co-solvent mixtures [14], but floating on water [1]. For example, the 1,3-dipolar cycloadditions between diethyl acetylenedicarboxylate and diazido-cyclohexanediols proceed best in pure water [1]. When the water is omitted so that the above reactants are mixed neat, the reactions are much slower and less selective, and, on a larger scale, become dangerous, because click chemistry reactions are, by definition, highly exothermic. The presence of water in these reactions is beneficial, not just for reactivity reasons, but also because water is the best heat-sink for handling the enormous heat output when click reactions are performed on larger scales. Yet another advantage of water as a reaction solvent is that its presence prevents interference from simple protic functional groups, like alcohols and amides, which are ubiquitous in biologically active organic molecules [15].

Huisgen's 1,3-dipolar cycloaddition of alkynes and azides yielding triazoles is, undoubtedly, the premier example of a click reaction [7]. Azides and alkynes are easy to install, and, despite being among the most energetic species known, they are also among the least reactive functional groups in organic chemistry. This stability, being purely kinetic in origin, is responsible for the slow nature of the cycloaddition reaction and the inertness of these functional groups towards biological molecules and towards the reaction conditions inside living systems (i.e. aqueous, and mild reducing environments). With the recently discovered dramatic rate acceleration of the azide–alkyne coupling event under copper-(I) catalysis [16,17], and the beneficial effects of water [16], this unique connection process seems to be redefining the concept of 'a perfect' reaction (Figure 1b). Given this new process, and the ready availability of the starting materials, highly diverse, unambiguous libraries become available quickly. There are no protecting groups, and with complete conversion and selectivity for the 1,4-disubstituted 1,2,3-triazole, structural uncertainties do not exist, rendering purification unnecessary.

The transformation is especially relevant for drug discovery, not just because of its reliability as a linking reaction, but also because of the favorable physicochemical properties of triazoles. The latter serve as rigid linking units that

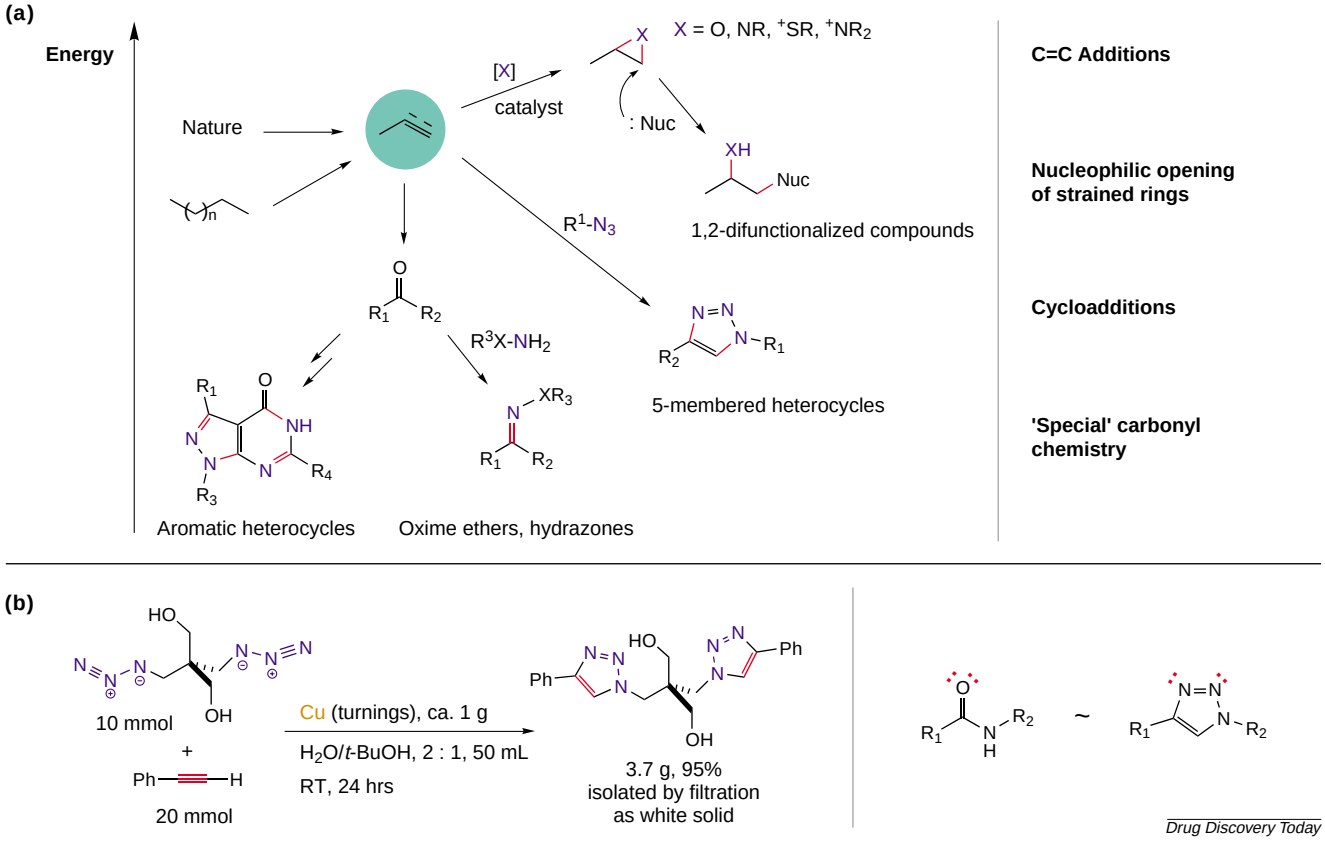


**Figure 1. (a)** Click Chemistry – energetically highly favorable linking reactions [1]. Unsaturated compounds provide the carbon framework. New groups are attached via carbon-heteroatom bonds (shown in red); **(b)** The premier click chemistry reaction: The copper-(I)-catalyzed coupling of azides and terminal acetylenes creates 1,4-disubstituted 1,2,3-triazole linkages, which share useful topological and electronic features with nature's ubiquitous amide connectors. However, unlike amides, triazoles are not susceptible to cleavage.

place the carbon atoms, attached to the 1,4-positions of the 1,2,3-triazole ring, at a distance of 5.0 Å (C-α distance in amides: 3.8 Å). In contrast to amides, triazoles cannot be cleaved hydrolytically or otherwise, and unlike benzenoids and related aromatic heterocycles, they are almost impossible to oxidize or reduce. They possess a large dipole moment of ~5 Debye (by *ab initio* calculation, RHF/6–311G**; cf. N-methyl acetamide: 3.7 – 4.0 Debye [18]), and nitrogen atoms two and three function as weak hydrogen bond acceptors.

## Click chemistry and drug discovery

Click chemistry is being used increasingly in biomedical research, ranging from lead discovery and optimization, to tagging of biological systems, such as proteins, nucleotides and whole organisms. The potential of this approach is highlighted here, by reviewing several early applications.

### *Synthesis of lead discovery libraries*

Over the course of five years, click chemistry laboratories at Coelacanth Corporation (now Lexicon Pharmaceuticals; http://www.lexicon-genetics.com/pharma) have employed solution-phase chemistry to produce a variety of screening libraries, containing a total of 200 000 individual compounds, each more than 85% pure and available in 25–50 mg amounts [19]. In line with the click chemistry philosophy, each library compound was produced in only one or two synthetic steps, from key building block reagents, using automated liquid handling workstations. Despite the short synthetic sequences, much compound diversity and novelty was achieved by starting with non-commercial building block reagents, prepared in-house on multi-gram or, even, kilogram scales. Examples include 'spring-loaded' epoxides and aziridines for the formation of 1,2-difunctionalized compounds by nucleophilic opening [20], imidoesters for the generation of five-membered aromatic heterocycles [21], azides for the synthesis of 1,2,3-triazole-derived libraries via 1,3-dipolar cycloaddition with β-ketoesters [22], and 3-aminoazetidines for the preparation of non-aromatic heterocyclic libraries [23]. Also, targeted libraries were made, one of which led to the discovery of

potent Peroxisome Proliferator-Activated Receptor γ (PPAR-γ) agonists [24].

The copper-(I)-catalyzed formation of 1,2,3-triazoles has recently been used to prepare functionalized resins for the solid phase synthesis of a library of dopaminergic arylcarbamides [25]. In another resin-based approach, Yli-Kauhaluoma *et al.* prepared 1,2,3-triazoles via thermal 1,3-dipolar cycloaddition of polymer-bound azides to alkynes, followed by cleavage from the solid support with TFA [26].

### *Drug discovery approaches based on the copper-(I)-catalyzed formation of triazoles from azides and acetylenes.*

With the ~$10^6$-fold rate acceleration of the copper-(I)-catalyzed variant [16] of Huisgen's 1,3-dipolar cycloaddition reaction [7], the generation of screening libraries has reached a new level of simplicity. Two subunits are reliably joined together by formation of a 1,4-disubstituted 1,2,3-triazole linkage. This ligation process works best in aqueous media without requiring protecting groups for any of the most common functional groups, enabling compound screening straight from the reaction mixtures (i.e. without prior purification).

*(a) Synthesis of neoglycoconjugates.* Carbohydrates play a central role in metabolism, cell–cell interaction and cell migration processes, and pathogen defence, offering a host of attractive drug discovery opportunities [27]. Despite this great potential, carbohydrate-based drug discovery is still in its infancy. Carbohydrates make poor lead compounds, owing to their notoriously modest affinities for the respective receptors or enzymes, poor pharmacological properties, and difficult syntheses. Click chemistry, in particular, the copper-(I)-catalyzed ligation of azides and acetylenes, promises to greatly simplify and accelerate the discovery of high-affinity carbohydrate mimetics.

Inspired by the way nature increases weak carbohydrate–receptor interactions through multivalency [28], Santoyo-Gonzalez and co-workers prepared a series of multivalent, triazole-linked neoglycoconjugates, using the robust copper-catalyzed coupling of carbohydrate-derived acetylenes and azides (Figure 2a) [29]. Complete regiochemical control and yields of greater than 80% were achieved using organic-soluble copper-(I) complexes as catalysts. Microwave irradiation considerably shortened the reaction times from several hours to a few minutes at room temperature. 'Disaccharides', derived from mannose, were prepared, and multiple mannose units were linked to aromatic and heteroaromatic cores. Even heptavalent manno-β-cyclodextrins were accessible with this approach.

*(b) Fucosyltransferase inhibitors.* Cell surface glyco-proteins and glyco-lipids bearing the sialyl Lewis$^x$ and sialyl Lewis$^a$ tetrasaccharide epitopes mediate a variety of crucial cell–cell recognition processes, such as fertilization, embryogenesis, lymphocyte trafficking, immune response and cancer metastasis [30]. The final step in the biosynthesis of these carbohydrates, catalyzed by fucosyltransferases, involves the transfer of an L-fucose moiety from guanosine diphosphate β-L-fucose (GDP-fucose) to a specific hydroxyl group of sialyl N-acetyl-lactosamine. Selective inhibitors of these enzymes might provide drugs by blocking the synthesis of fucosylated end-products, and the pathology they trigger. However, success in this field has been limited, thus far, with the best inhibitors in the micromolar range [31,32]. Wong *et al.* identified nanomolar inhibitors from a compound library that was prepared by linking a GDP-derived acetylene to a library of azides, using the copper-(I)-catalyzed triazole formation (Figure 2b) [15]. The excellent yields and the absence of protecting groups allowed 85 test compounds to be rapidly prepared in water and screened straight from the reaction mixture. Hit follow-up, conducted on purified compounds against a panel of fucosyl and galactosyl transferases and kinases, revealed biphenyl derivative 1 as the most potent inhibitor of human α-1,3-fucosyltransferase VI that has been found to date, and it was also revealed to be selective for this one enzyme.

*(c) Development of HIV protease inhibitors.* HIV protease is responsible for the final stages of virus maturation and, thus, its inhibitors are useful drugs for the treatment of AIDS [33]. The emergence of drug-resistant mutant HIV proteases increases the demand for new inhibitors [34]. Wong *et al.* prepared two focused libraries of 50 compounds each, based on hydroxyethylamine peptide isosteres (Figure 2c) [35]. Azide-bearing scaffolds 2 and 3, inspired by Glaxo's Amprenavir (http://www.gsk.com) [36], were united via the new copper-catalyzed process with acetylenes, for library production. These libraries, which were already in aqueous solution from the synthesis step, were used directly, for screening against wild type HIV-1 protease and three mutants (G48V, V82F, V82A). As was predicted by molecular modeling, compounds derived from scaffold 2 did not yield any hits at 100 nM concentration, whereas, scaffold 3 provided four hit compounds with good activity at 10 nM concentration. Two of these compounds strongly inhibited all four proteases tested, with activities in the low nanomolar range (purified compounds).

## In situ *click chemistry*

Mock's discovery of a dramatic rate acceleration of the azide–alkyne cycloaddition by sequestering the two components inside a host structure [37–39], prompted Sharpless *et al.* to investigate a new paradigm for drug discovery, which is dependent on irreversible, target-guided synthesis

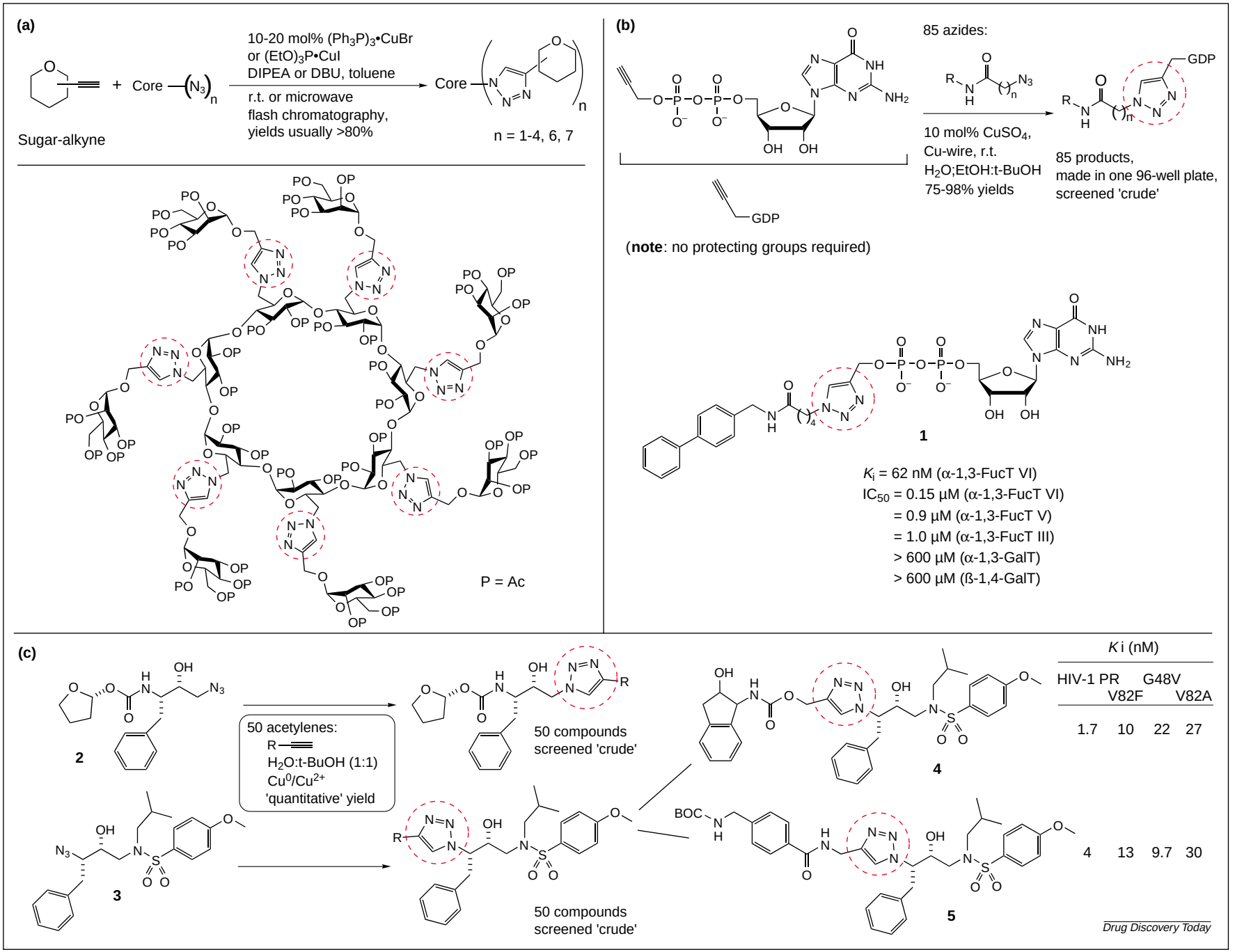
(a)
Sugar-alkyne
10-20 mol% (Ph3P)3•CuBr
or (EtO)3P•CuI
DIPEA or DBU, toluene
r.t. or microwave
flash chromatography,
yields usually >80%
n = 1-4, 6, 7
P = Ac
(b)
85 azides:
10 mol% CuSO4,
Cu-wire, r.t.
H2O;EtOH:t-BuOH
75-98% yields
85 products,
made in one 96-well plate,
screened 'crude'
(note: no protecting groups required)
1
Ki = 62 nM (α-1,3-FucT VI)
IC50 = 0.15 μM (α-1,3-FucT VI)
= 0.9 μM (α-1,3-FucT V)
= 1.0 μM (α-1,3-FucT III)
> 600 μM (α-1,3-GalT)
> 600 μM (ß-1,4-GalT)
(c)
2
3
50 acetylenes:
H2O:t-BuOH (1:1)
Cu0/Cu2+
'quantitative' yield
50 compounds
screened 'crude'
50 compounds
screened 'crude'
4
5
Ki (nM)
HIV-1 PR G48V V82F V82A
1.7 10 22 27
4 13 9.7 30
Drug Discovery Today

**Figure 2.** Application of the copper-(I)-catalyzed triazole formation in drug discovery; **(a)** multivalent neoglycoconjugates (P = acetyl) [29]; **(b)** human a-1,3-fucosyl transferase VI inhibitors [15]; **(c)** HIV-protease inhibitors [35]. The crude products in examples (b) and (c) were sufficiently pure for screening straight from the reaction mixture.

of high-affinity inhibitors from reagents that are inert under physiological conditions [40]. By contrast, other approaches employ highly reactive reagents (e.g. aldehydes and hydrazines; thiols and α-chloroketones etc.) and reversible reactions for the *in situ* assembly of inhibitors inside a target's binding pocket [41–44]. Acetylcholine esterase (AChE) was chosen as the target. Its inhibitors have been employed for over a century in various therapeutic regimens and to investigate the role of acetylcholine in neurotransmission [45,46]. The enzyme's active site is located at the base of a narrow gorge, ~20 Å in depth [47]. A second, peripheral binding site exists at the rim of the gorge, near to the enzyme surface [48,49]. The concerted thermal 1,3-dipolar cycloaddition reaction between azide and acetylene reagents (which carry active-site and peripheral-site binding groups via flexible spacers) was selected for this study for several reasons. First, the reaction is extremely slow at room temperature, second, it does not involve components that might disturb the binding sites (external reagents, catalysts, by-products), and third, the reactants are bio-orthogonal. A substantial rate acceleration was observed for certain azide–acetylene reagent combinations in the presence of the enzyme. From 49 building block combinations, the enzyme selected the TZ2/PA6 pair, leading to the formation of a sole reaction product in a highly regioselective fashion: the TZ2PA6 syn-6 triazole (Figure 3). By contrast, chemical synthesis in the absence of enzyme provided a roughly 1:1 mixture of syn- and anti-6 regioisomers. Both are respectable inhibitors, but the syn-6 isomer, with a 100-fold greater affinity and a sub-picomolar dissociation constant for certain acetylcholine esterases, has a potency greater than all known non-covalent organic AChE inhibitors. Thus, AChE itself, served as the reaction vessel, synthesizing its own inhibitor by equilibrium-controlled sampling of various possible pairs of reactants in its gorge until the irreversible cycloaddition between azide and acetylene essentially 'froze' the pair that fits best into the binding pocket. A recent X-ray crystallographic analysis of both the syn-6- and anti-6-mouse AChE complexes at 2.45–2.65 Å resolution, reveals that the former has effectively trapped the enzyme in a previously unknown conformational state [50,51]. If this

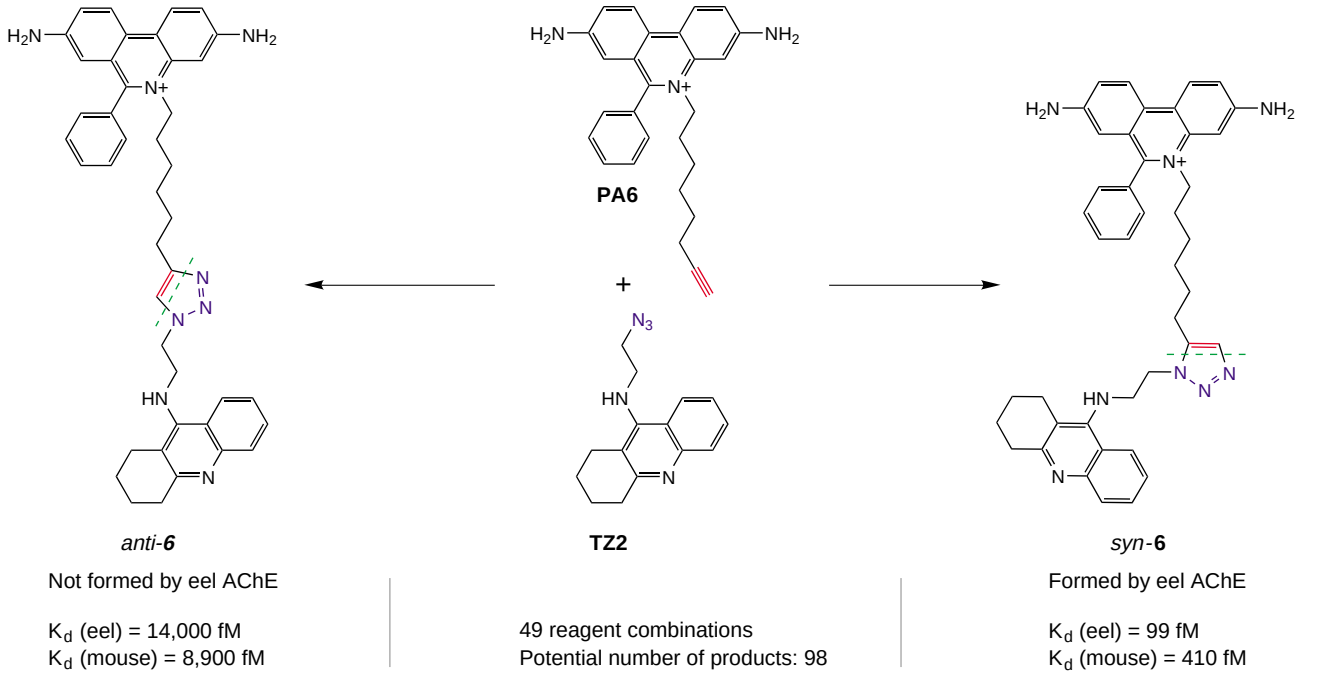


**Figure 3**. *In situ* click chemistry [40]. The enzyme AChE catalyzes the formation of its own inhibitor. Whereas the less active anti-triazole (anti-6), which is not formed *in situ*, does not alter the conformational state of the enzyme, the highly potent syn-triazole (syn-6), formed *in situ*, traps the enzyme in a 'new' conformational state, which is probably a low abundance one in the absence of syn-6.

'open' conformer is in facile equilibrium with the ground state, it might help to explain the enormous turnover rates of acetylcholine esterases – similar to a breathing motion that is in pace with the catalytic cycle. In addition, this study revealed that the 1,2,3-triazole cores interact strongly with the protein through hydrogen bonding to N2 and N3 and through their large dipole moments (~5 Debye).

## Click chemistry and bioconjugation

*In vivo* and *in vitro* bioconjugation applications benefit from the unprecedented reliability of the copper-catalyzed azide–acetylene union, the inertness of the reactants under physiological conditions, and the mild reaction conditions.

### *Tagging of live organisms and proteins*

The application of mild methods for the chemical modification of components in, or on, living cells under physiological conditions has been pioneered by the Bertozzi group [52,53]. Finn *et al.* recently succeeded in using the new copper-(I)-catalyzed 1,2,3-triazole formation for labeling intact Cowpea mosaic virus particles (CPMV) with fluorescein (Figure 4a) [54]. CPMV's capsid is made up of 60 copies of an asymmetric two-protein unit, which encapsulate the single-stranded RNA genome. Each unit contains one reactive Cys and Lys. To these, via traditional bioconjugation methods, a total of 60 azides per virus particle were attached. Under optimized conditions, all 60 azide groups reacted to form triazoles, for a yield of the 60-mer of >95%.

Tirrell and Link have recently disclosed how, using an ingenious sequence of molecular biology techniques, they forced *Eschericia coli* cells into making and displaying an azide-bearing outer membrane protein C (OmpC), in which methionine residues were replaced by azidohomoalanine 7 [55]. These bacterial cells, now presenting azide groups to the extracellular milieu, were successfully biotinylated, under the special copper-catalyzed conditions developed by the Finn group for their virus case, with a biotin-alkyne reagent.

The Schultz laboratory then reported that their method for genetically-encoded incorporation of the azide and acetylene tyrosine analogs 8 and 9 into proteins of *Saccharomyces cerevisiae*, could be followed up by capture of dyes, using the copper-(I)-catalyzed azide–alkyne triazole-coupling, as optimized by Finn *et al.* [54] for *in situ* bioconjugations [56].

### *Activity-based protein profiling (ABPP)*

ABPP is a chemical method that employs active site-directed probes to tag proteins and monitor their expression levels and function in complex proteomes [57,58]; however, the bulky reporter tags that are currently used require cell homogenization before analysis, thereby, preventing measurements in living organisms. Cravatt *et al.* have solved this problem with small, cell-permeable reagents that carry an azide group for later dye attachment via the bio-orthogonal, copper-(I) catalyzed reaction with acetylenes (Figure 4b) [58]. The authors detected glutathione S-transferases (GSTO 1–1), aldehyde dehydrogenases (ALDH-1) and enoyl CoA hydratases (ECH-1) at endogenously expressed levels in viable cells, by incubation with 6-azidohexyl benzenesulfonate (PS-$N_3$), followed by homogenization and tagging with Rhodamine-acetylene (Rh-acetylene). Quantification of GSTO 1–1 levels in breast cancer cell lines yielded results that were comparable with traditional methods. The Cravatt approach works even in live animals, allowing ECH-1 to be observed in the heart muscle of mice one hour after injection with PS-$N_3$ and 'staining' of the unique protein from the crude heart-homogenate, via copper-catalyzed conjugation with Rh-acetylene. By enabling the determination of protein expression levels in living organisms, this new *in vivo* ABPP method provides more unbiased results, because tagging occurs before cell death.

### *Labeling of DNA*

Ju *et al.* prepared primers for the Sanger dideoxy chain termination reaction for DNA sequencing [59], by tagging the M13–40 universal forward sequencing primer at its 5′-end, with alkynyl 6-carboxyfluorescein (FAM), using the concerted thermal 1,3-dipolar cycloaddition process (Figure 4c) [60]. The advantages of this method over existing ones [61–64] are that it proceeds in high yield under relatively mild conditions, without the need for additives (e.g. catalysts or reagents) or the formation of by-products, rendering purification of the labeled single-stranded DNA unnecessary. The FAM-triazole-M13–40 primer was successfully used in the Sanger method, terminating with biotinylated dideoxyATP (ddATP-Biotin), to produce DNA sequencing fragments, using PCR-amplified DNA as a template. The DNA fragments were analyzed by capillary array electrophoresis, the peaks representing the FAM fluorescence emission from each DNA fragment that was extended from the fluorescence-labeled primer and terminated by ddATP. The 'A' sequencing ladder that was obtained in this way matched, exactly, the sequence of the DNA template.

## Conclusions

In summary, click chemistry has proven to be a powerful tool in biomedical research, ranging from combinatorial chemistry and target-templated *in situ* chemistry for lead discovery, to bioconjugation strategies for proteomics and

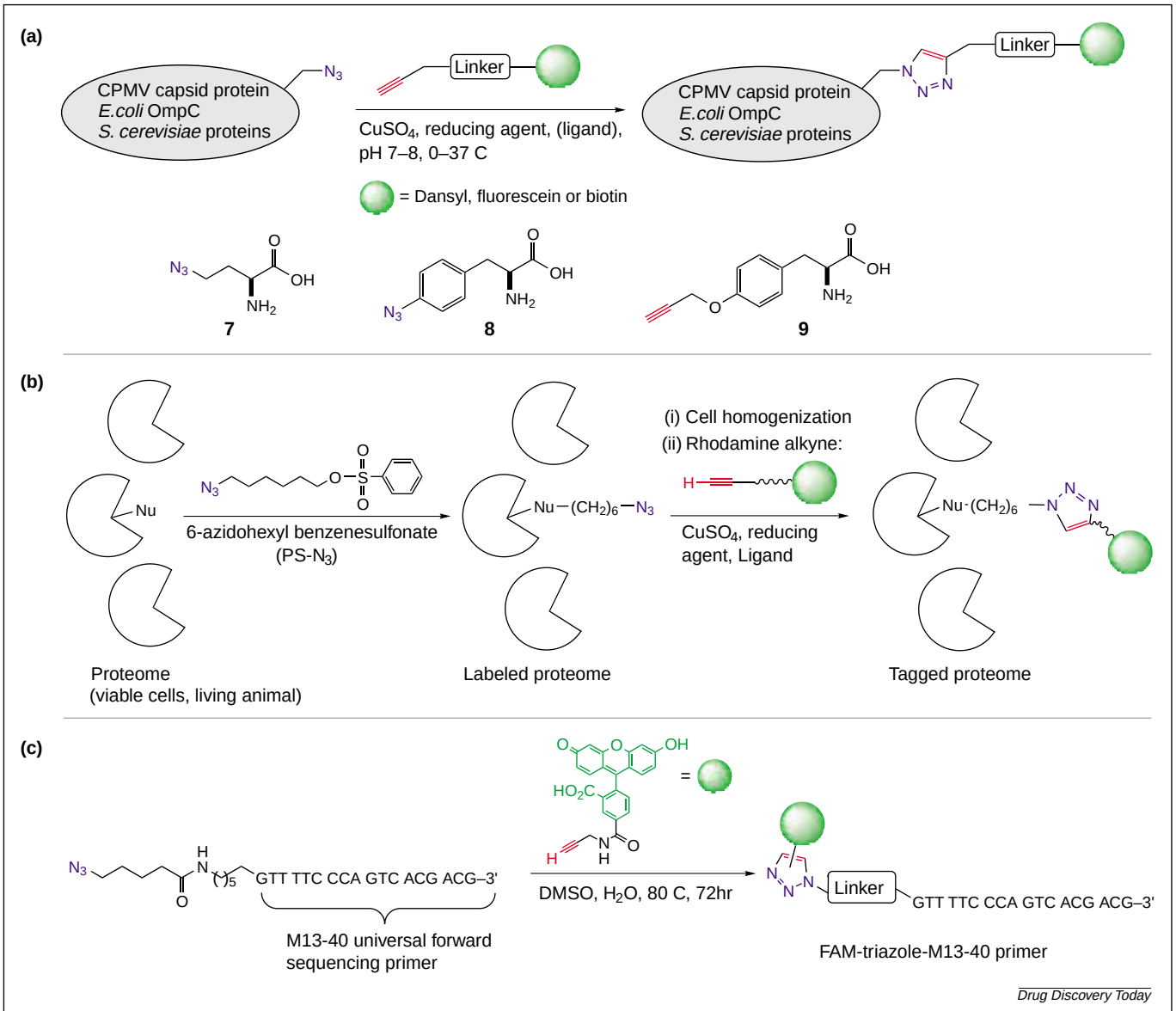


**Figure 4**. Bioconjugation applications using the cycloaddition of azides and acetylenes; **(a)** virus, cell and protein tagging [54–56]; **(b)** *in vivo* activity-based protein profiling [58]; **(c)** DNA labeling to enable sequencing by the Sanger dideoxy chain termination reaction [60].

DNA research. Of the various click chemistry reactions that are available to us, the union of azides and acetylenes to give triazoles deserves special recognition. Azides and acetylenes are stable across a broad range of organic reaction conditions and in biological environments, yet they are highly energetic functional groups. Their irreversible combination to triazoles is highly exothermic, albeit slow. The full potential of this ligation reaction was unleashed with the discovery of copper-(I) catalysis. Benefiting from more than a million-fold rate acceleration, this process proceeds in near-quantitative yields in water, and because no protecting groups are used, the products are screened directly from the reaction mixture. This triazole-forming process, and click chemistry in general, promise to accelerate both lead finding and lead optimization, due, above all, to its great scope, modular design, and reliance on extremely short sequences of near-perfect reactions.

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