VIP Click Chemistry Very Important Paper

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Discovery of Chemoselective and Biocompatible Reactions Using a High-Throughput Immunoassay Screening**

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The discovery of new chemical reactions is a long-standing goal of organic chemists. For decades, synthetic problems motivated the development of new methodologies to continuously expand the reaction toolkit in organic synthesis. As alternatives to purely rational approaches, strategies that offer more room for serendipity have recently emerged. In these approaches, the discovery is a result of the systematic exploration of a large number of chemical reactions through the use of robust high-throughput screening methods based either on mass spectrometry techniques[1] or on DNA technologies.[2] Although this strategy was already proven to be efficient with the discovery of several new interesting reactions,[3] this does not guarantee the potential impact of the discovered reactions. A more powerful approach would be the increase of the level of selection in a manner that only powerful reliable reactions are discovered. Such a highly demanding selection should therefore be based not only on reaction efficiencies, but also on other parameters that would ensure the usefulness of the discovered reaction. In 2001, K. B. Sharpless introduced the concept of "click chemistry", which has been widely and successfully applied since then, and listed a series of important criteria that may influence the extent and the impact of a chemical reaction.[4] Among them, chemoselectivity, simplicity of reaction conditions, and high efficiency, even in complex media, are probably the most important ones. This can be highlighted by the startling number of applications in organic synthesis, materials science, and biotechnology of the copper-catalyzed alkyne–azide cycloaddition reaction (CuAAC), which is one of the most powerful click reactions described to date.[5]

Herein, we disclose an approach to accelerate the discovery of such important chemical reactions through the use of an immunoassay technique. As we previously described,[6] sandwich immunoassays can be successfully applied to monitor cross-coupling reactions by connecting small-molecule tags to chemically reactive groups. Products of bond-forming coupling reactions can then be specifically detected by two specific antitag monoclonal antibodies (mAbs) using standard ELISA techniques: one mAb captures the double-tagged coupling product on a solid phase and a second acts as a detector. We recently showed that the throughput of this adapted immunoassay (typically around 1000 analyses per day and person) allows the fast identification of new reactions among thousands of combinations of reactive functions and catalysts.[7] One crucial advantage of this screening method relies on the high specificity of mAbs, permitting the precise and sensitive quantification of the double-tagged cross-coupling products in complex mixtures without work-up. Here, we decided to fully exploit this advantage by designing a series of successive screening in order to identify new, efficient, chemoselective, and biocompatible [3+2] cycloaddition reactions. Our approach (Figure 1) involves three main steps: 1) reactions of tagged dipoles, dipolarophiles, and transition metals, run in 96-well plates in a parallel manner, 2) immunoassays to identify active combinations, to optimize them, and to assess their kinetics, chemoselectivity, and biocompatibility, and 3) validation of hits by reproducing the reactions in flasks with nontagged functions.

The core experiment was conducted with 11 tagged dipoles and 8 tagged dipolarophiles. All of these reactants were selected for their chemical stability and easy synthetic access, as simple reaction conditions that involve readily available reactants are a key requirement for powerful

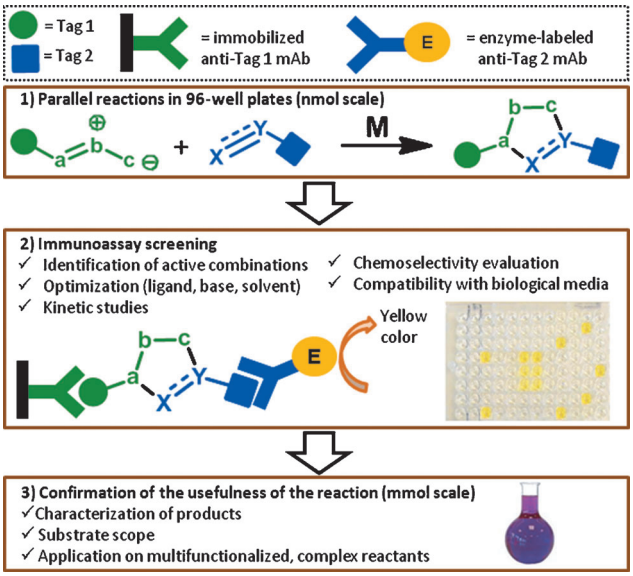

Figure 1. Approach to the discovery of valuable reactions.

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chemical processes. In a parallel manner, reactants were combined and exposed to one set of reaction conditions in the presence of one equivalent of one of the 31 transition metals that were tested. Control experiments in the absence of metal were also carried out in parallel. Reactions were run at 50 °C in 20 μL (total volume) of DMF containing 100 nmol of each reactant, leading to a total of 2816 parallel reactions performed in 96-well plates. The yields of the crudes were measured by immunoassay using a calibration curve obtained with a double-tagged reference product (see Figures S4 and S5). All combinations were screened within two days, highlighting 51 coupling reactions (Figure 2C). Among these, 9 were well-known reactions, such as the Cu-catalyzed azide–alkyne (**3C**),[8] azide–bromoalkyne (**3F**),[9] and azomethine imine–alkyne (**11C**) cycloadditions.[10] Under our reaction conditions, yields were poor throughout and did not exceed 15 %. Optimizations of the 42 new reactions were thus carried out by assaying each reaction with 4 different metal salts, 8 ligands, 4 bases, and 8 solvents. All optimized reactions were then systematically tested for their kinetic properties, chemoselectivity, and compatibility with biological constituents. In short, reactions were performed first in the presence of one

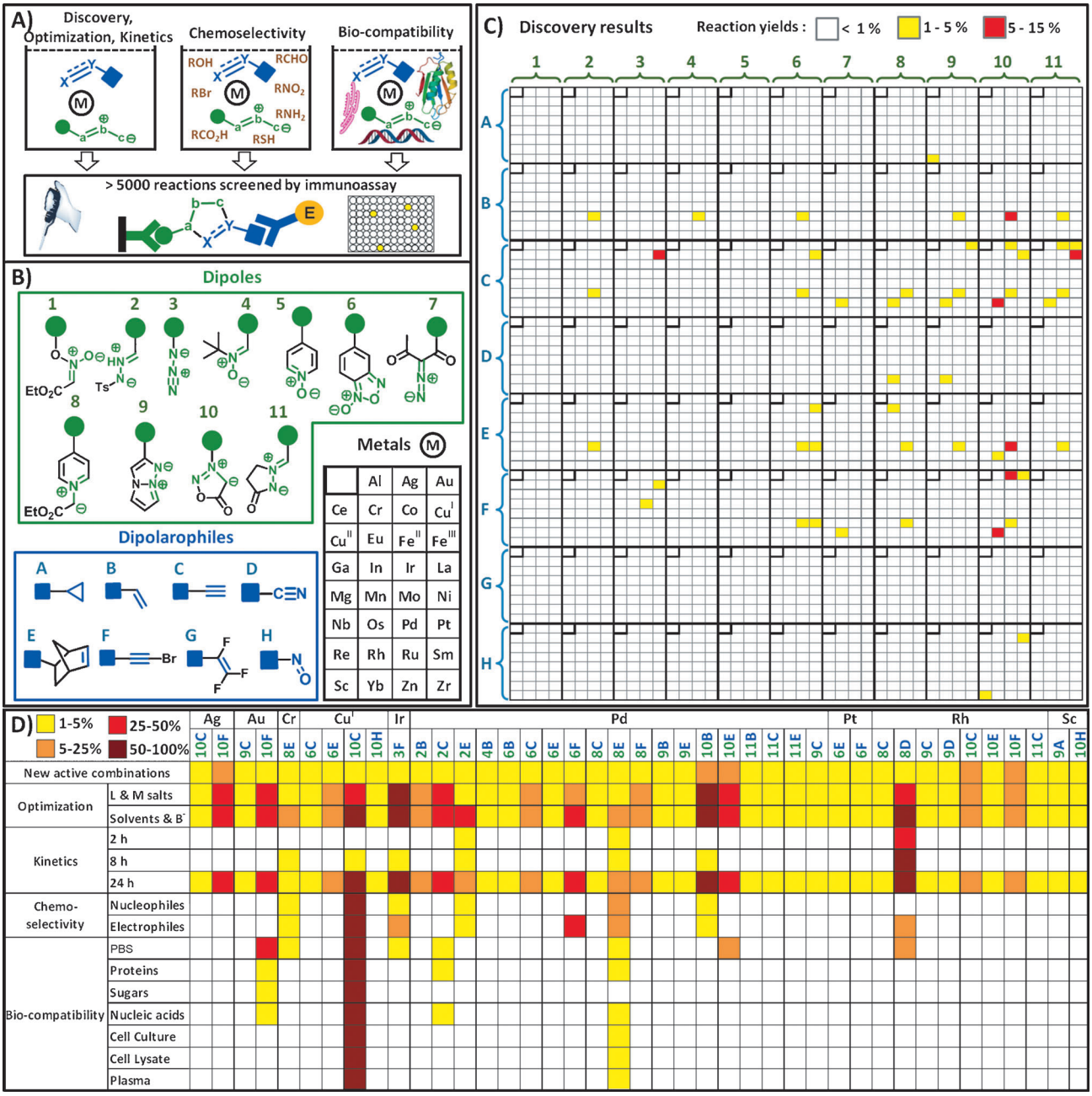

Figure 2. A) Global screening strategy, B) libraries of dipoles, dipolarophiles, and metals, C) reaction discovery, D) optimization and evaluation of the reaction properties. Reactions were carried out with 1.25 mM reactants, 1 equiv of metal (M), 1 equiv of ligand (L), 1 equiv of base (B^-) in 20 μL total volume. Discovery and optimizations were carried out at 50 °C for 24 h; kinetics and chemoselectivity experiments at 25 °C in organic solvents; biocompatibility experiments at 37 °C in aqueous media. PBS = phosphate-buffered saline.

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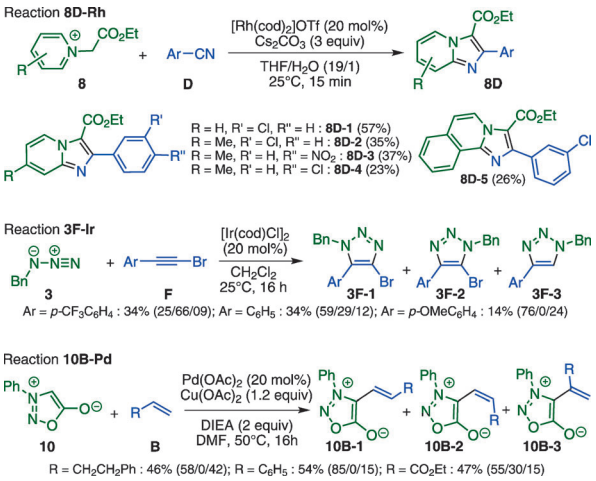
equivalent of nucleophiles (amine, thiol, acid, etc.) or electrophiles (Michael acceptor, bromoalkane, aldehyde, etc.) and second in the presence of concentrated solutions of proteins, DNA, or sugars, in cell media as well as in blood plasma. The yields of the crudes were then determined by sandwich immunoassay. The yields of about half of the 42 hits were significantly increased, but only those of 4 reactions (**3F**-Ir, **8D**-Rh, **10B**-Pd, and **10C**-Cu) were optimized to more than 50% (Figure 2D).

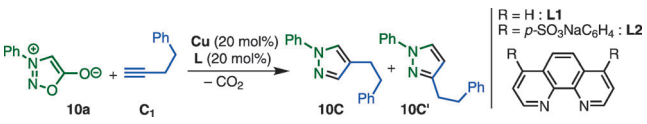
Three of these reactions were only of limited interest in terms of chemoselectivity and bioorthogonality, but were nevertheless further explored on the mmol scale using nontagged reactants and catalytic amounts of metals (Scheme 1). We first focused our efforts on reaction **8D**-Rh, which apparently displayed the fastest kinetics in well plates according to the screening results. The reaction was reproduced in a flask and was indeed complete in only 15 minutes at room temperature using cationic rhodium (20 mol%) to generate imidazolopyridine compounds through a 1,3-dipolar cycloaddition of pyridinium salts with aromatic nitriles. Only traces of product were detected in the absence of rhodium. However, yields were moderate due to the fast degradation of the pyridinium substrates under our reaction conditions. Reactions of azides with bromoalkynes in the presence of the iridium dimer $[Ir(cod)Cl]_2$ generated a mixture of 1,4 and 1,5 regioisomers of bromotriazoles and dehalogenated 1,4-triazole in various ratios with poor yields, depending of the electronic enrichment of the alkyne. Exploration of reaction **10B**-Pd revealed a new dehydrogenative Heck coupling of sydnones with alkenes, allowing a C–H/C–H cross-coupling at only 50°C, but with moderate yields and low level of regioselectivity.

The improvement of these reactions would merit further investigation, but at this point our interest was entirely focused on the reaction involving dipole **10**, dipolarophile **C**, and copper(I) salts. Indeed, according to the screening results in Figure 2D, this particular combination appeared to be the most efficient, chemoselective, and bioorthogonal of the 2816



Scheme 1. Interesting reactions identified by high-throughput screening (yields of isolated products). cod = cycloocta-1,5-diene, DIEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, Tf = trifluoromethanesulfonyl.

Table 1: Reaction **10C**-Cu.[a]



Entry	Cu/**L**	Conditions	Yield [%]	**10C**/**10C'**
1	–	DMF, Ar, 153°C	15	25/75
2	CuI/**L1**	DMF, Ar, 60°C	92	100/0
3	$CuSO_4$/AS[b]/**L1**	*t*BuOH/H_2O, air, 60°C	99	100/0
4	$CuSO_4$/AS[b]/**L2**	H_2O, air, 60°C[c]	99	100/0
5	$CuSO_4$/AS[b]/**L2**	*t*BuOH/H_2O, air, 25°C	67	100/0

[a] Experiments were carried at a concentration of 0.1 M with 1 equiv of reactants and 1 equiv of TEA for 14 h. [b] 2 equiv of AS. [c] 1 equiv of $N(CH_2CH_2OH)_3$ in place of TEA. AS = sodium ascorbate, TEA = triethylamine.

combinations we tested. We first examined this reaction using phenylsydnone **10a** and alkyne $\mathbf{C_1}$ as model substrates (Table 1). In accordance with the optimization data obtained during the screening procedure, the reaction proceeded efficiently under Cu^I–phenanthroline catalysis at room temperature or with gentle heating to produce pure 1,4-pyrazole **10C**. The thermal 1,3-dipolar cycloaddition of sydnones with alkynes, known since Huisgen's work in 1962,[11] has been of limited success over the years because of harsh conditions and low regioselectivity, which generates a mixture of pyrazoles.[12] Addition of Cu^I–phenanthroline complexes allows the exclusive formation of 1,4-pyrazoles under milder conditions (compare entry 1 with entries 2–5 in Table 1).

Convinced by its synthetic impact, we investigated the substrate scope of this new copper-catalyzed reaction (Scheme 2). The reaction was remarkably tolerant to all tested functional groups that were present either in the sydnone or the alkyne substrate.

For example, dansylated peptide **10C-20** was synthesized quantitatively in water using this reaction. Although this Cu-catalyzed sydnone–alkyne cycloaddition (CuSAC) reaction has some limitations (reaction was unsuccessful with N-alkyl sydnones), it has many advantageous features. The reaction proceeded smoothly in many solvents, including biological media, giving clean reactions with no trace of by-product. For example, **10C-13** was synthesized with 88% yield at 37°C in pure human blood plasma.

With the reaction scope established, we next sought to demonstrate the usefulness of the CuSAC reaction for bioconjugation applications. BSA–sydnone conjugate, obtained through standard peptide coupling using an excess of sydnone **10b**, was reacted with 1.5 equivalents (per sydnone) of dansylated alkyne $\mathbf{C_2}$ under CuSAC conditions. According to MALDI-TOF analysis, 75% of the sydnones on BSA were transformed into pyrazoles, and sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) analysis confirmed the effective dansylation of the protein (Figure 3).

The CuSAC reaction might be considered a new example of a highly effective and selective transformation that results from the reactivity of in situ generated copper(I) acetylides. A possible mechanism may involve coordination of N2 of the

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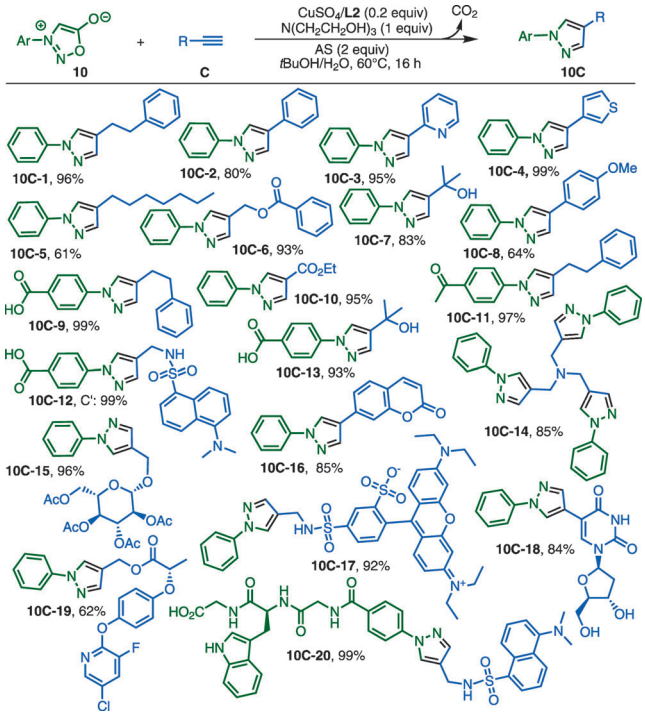
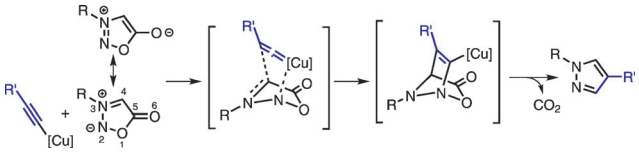


Scheme 2. Scope of the CuSAC reaction (yields of isolated products).

sydnone substrate to the copper center of the acetylide, thus provoking an increase of the nucleophilicity of the triple bond and of the electrophilicity of C4 of the sydnone, ultimately resulting in the formation of the C–C and C–N bonds (Scheme 3).

We have shown that the exploration of a designed series of reactions with a powerful high-throughput screening, which allows the evaluation not only of the reaction efficiencies but also of the chemoselectivity, speed, and biocompatibility, is a valid approach to the discovery of new chemical transformations with high potential. We explored 1,3-dipolar cycloadditions, which in theory represent a well-known chemical reactivity space. A rapid survey of the literature



Scheme 3. Proposed mechanism for the CuSAC reaction.

surprisingly shows that less than 10% of the dipole/dipolarophile/metal combinations tested in this study were previously reported. From the 90% of the remaining combinations, a reaction that generates pyrazoles with high efficiency, chemoselectivity, and biocompatibility was discovered and should be a useful addition to the list of click reactions. We think that such an approach that is based on systematic evaluation of reaction properties by high-throughput screening could be advantageously applied to identify new important chemical transformations in the future.

Experimental Section

Typical procedure for the CuSAC reaction: A freshly prepared aqueous solution (2 mL) of $CuSO_4 \cdot 5H_2O$ (50 mg, 0.2 mmol), ligand **L2** (0.2 mmol), and triethanolamine (1 mmol) was added to a solution of sydnone (1 mmol), alkyne (1 mmol), and sodium ascorbate (2 mmol) in a mixture of *t*BuOH/H_2O (11:9, 1.8 mL). The resulting mixture was stirred at 60 °C for 16 h, then quenched with a solution of HEDTA (0.5 M, 2 mL) and extracted with CH_2Cl_2. Combined organic layer was dried over Na_2SO_4 and concentrated to afford the desired product.

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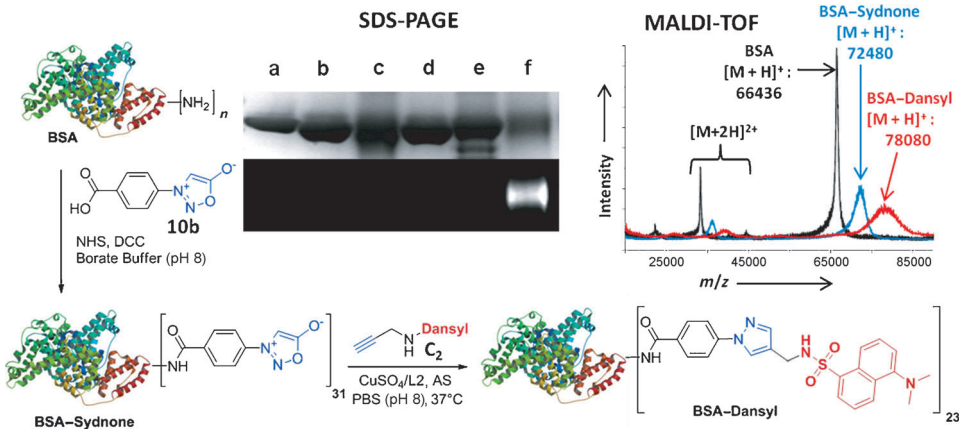

Figure 3. Dansylation of BSA using the CuSAC reaction. SDS-PAGE: the upper images show staining with Coomassie blue; the lower images show the gels visualized under ultraviolet irradiation. Lane a: BSA, lane b: BSA–sydnone; lane c: without alkyne $\mathbf{C_2}$; lane d: without copper; lane e: without sydnone **10b**; lane f: BSA–dansyl. MALDI-TOF analysis of BSA, BSA–sydnone, and BSA–dansyl. DCC = 1,3-dicyclohexylcarbodiimide, NHS = *N*-hydroxysuccinimide.

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