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Bioconjugation with Strained Alkenes and Alkynes

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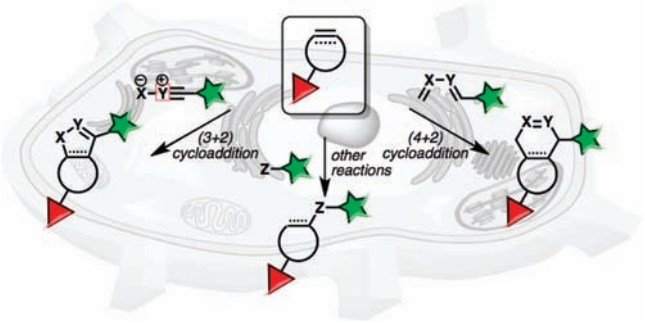

The structural complexity of molecules isolated from biological sources has always served as an inspiration for organic chemists. Since the first synthesis of a natural product, urea, chemists have been challenged to prepare exact copies of natural structures in the laboratory. As a result, a broad repertoire of synthetic transformations has been developed over the years. It is now feasible to synthesize organic molecules of enormous complexity, and also molecules with less structural complexity but prodigious societal impact, such as nylon, TNT, polystyrene, statins, estradiol, XTC, and many more.

Unfortunately, only a few chemical transformations are so mild and precise that they can be used to selectively modify biochemical structures, such as proteins or nucleic acids; these are the so-called bioconjugation strategies. Even more challenging is to apply a chemical reaction on or in living cells or whole organisms; these are the so-called bioorthogonal reactions. These fields of research are of particular importance because they not only pose a worthy challenge for chemists but also offer unprecedented possibilities for studying biological systems, especially in areas in which traditional biochemistry and molecular biology tools fall short.

Recent years have seen tremendous growth in the chemical biology toolbox. In particular, a rapidly increasing number of bioorthogonal reactions has been developed based on chemistry involving strained alkenes or strained alkynes. Such strained unsaturated systems have the unique ability to undergo (3 + 2) and (4 + 2) cycloadditions with a diverse set of complementary reaction partners. Accordingly, chemistry centered around strain-promoted cycloadditions has been exploited to precisely modify biopolymers, ranging from nucleic acids to proteins to glycans.

In this Account, we describe progress in bioconjugation centered around cycloadditions of these strained unsaturated systems. Being among the first to recognize the utility of strain-promoted cycloadditions between alkenes and dipoles, we highlight our report in 2007 of the reaction of oxanobornadienes with azides, which occurs through a sequential cycloaddition and retro Diels–Alder reaction. We further consider the subsequent refinement of this reaction as a valuable tool in chemical biology. We also examine the development of the reaction of cyclooctyne, the smallest isolable cyclic alkyne, with a range of substrates. Owing to severe deformation of the triple bond from ideal linear geometry, the cyclooctynes show high reactivity toward dienes, 1,3-dipoles, and other molecular systems. In the search for bioorthogonal reactions, cycloadditions of cyclic alkenes and alkynes have now established themselves as powerful tools in reagent-free bioconjugations.

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Introduction

The current state of art in organic chemistry enables the preparation of highly complex molecular structures, by application of a wide toolbox of synthetic transformations.[1] Unfortunately, the vast majority of chemical techniques are executed under strictly defined conditions, requiring toxic solvents, stoichiometric reagents, extreme temperatures, exclusion of moisture or oxygen, and with carefully designed protective group protocols. As a consequence, only a small subcategory of chemical transformations is suitable for modification of biomolecules (proteins, nucleic acids, sugars) that typically proceed in water and at near-ambient temperature (4−37 °C). Moreover, such biomolecular modification must be highly chemospecific in the sense that only a single functionality of interest (e.g., the lysine ε-amino group in proteins) is selectively modified in the presence of a plethora of other functional groups. Clearly, multiple reactions at identical or similar functional groups can hardly be avoided with such a strategy. A versatile solution to selective and single bioconjugation is found in so-called bioorthogonal chemistry, based on covalent bond formation between two abiotic groups with exclusive mutual reactivity (Figure 1A). Bioconjugation employing a bioorthogonal reaction typically involves a two-stage strategy: first, the introduction of one reactive component into a biomolecule (chemically or biochemically), followed by bioorthogonal conjugation (Figure 1B). In this Account, we will delineate the developments within the field of bioorthogonal reactions centered around cyclooadditions of strained unsaturated systems, alkenes, and alkynes, and in particular the application thereof in bioconjugation.

It may be argued that the concept of bioorthogonal chemistry saw the light with the advent of the Staudinger ligation: the first mild and highly selective reaction applicable to in vivo ligation of two abiotic groups.[2] In a seminal paper in 2000, Bertozzi and Saxon first demonstrated the power of the Staudinger ligation[2] in the fluorescent staining of azido-sugars residing on the cell surface of Jurkat or HeLa cells with a phosphine probe. Since then, the Staudinger ligation has found application in in vivo experiments, for example, cell surface remodeling, live-cell imaging, and the visualization of O-linked glycosylation in mice.[3]

Strained Alkenes

The Staudinger ligation paved the way for a conceptually new way of thinking, involving the translation of knowledge of chemical reactions to reactions in living systems. In this respect, particular inspiration is found in chemistry explored by Huisgen et al. involving cycloaddition reactions of unsaturated systems with 1,3-dipoles.[4] For example, Sharpless et al. identified Huisgen reactions as key tools for the concept of "click chemistry",[5] due to the large thermodynamic driving force. Biological application of dipolar cycloadditions, however, is limited due to the typically high activation energies, which necessitate heating above 50 °C. Fortunately, ring strain provides a solution to overcome this barrier.

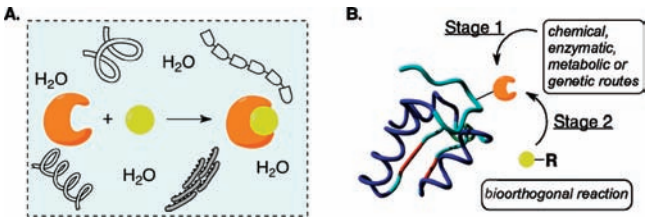


FIGURE 1. (A) Bioorthogonal reactions: full selectivity and inert to surrounding functionality. (B) Bioconjugation employing bioorthogonal chemistry.

Oxanorbornadienes. It was noted already by Alder and Stein[6] and Huisgen et al.[7] that strained alkenes, for example, norbornene, are over a hundred times more reactive toward dipoles than unstrained olefins.[8] We were among the first to recognize that strain-promoted cycloadditions between alkenes and dipoles (Figure 2) possess high value for bioconjugation. We envisioned that a combination of ring strain and electron deficiency,[9] as in oxa-bridged bicyclic systems **1**,[10] could lead to increased reactivity in (3 + 2) cycloadditions. More specifically, we found that cycloaddition of an azide (**2**) to **1** occurs preferentially at the electron-poor double bond with a reaction rate constant of 8.7×10^{-4} M^{-1} s^{-1}.[11] Conveniently, the intermediate triazoline adduct **3** undergoes a spontaneous retro-Diels−Alder reaction, with release of furan, leading to stable 1,2,3- and 1,4,5-triazoles **4**.

Several other investigators have appreciated the potential of strained bicyclic alkenes for bioconjugation. For example, Carell et al. reported that cycloaddition of norbornene to nitrile oxide **6**, proceeds effectively in a range of solvents, leading to a mixture of regioisomeric isoxazolines **7**.[12]

Another bioorthogonal ligation was reported by Hilderbrand and co-workers,[13] based on the inverse electron-demand Diels−Alder cycloaddition of tetrazines to norbornenes, followed by retro-Diels−Alder elimination of dinitrogen.[14] To this end, tetrazine **9**, equipped with an amino group for functionalization, was reacted with norbornene **8** with >93% conversion in aqueous buffer, affording the expected dihydropyridazines **10** (plus regioisomers).

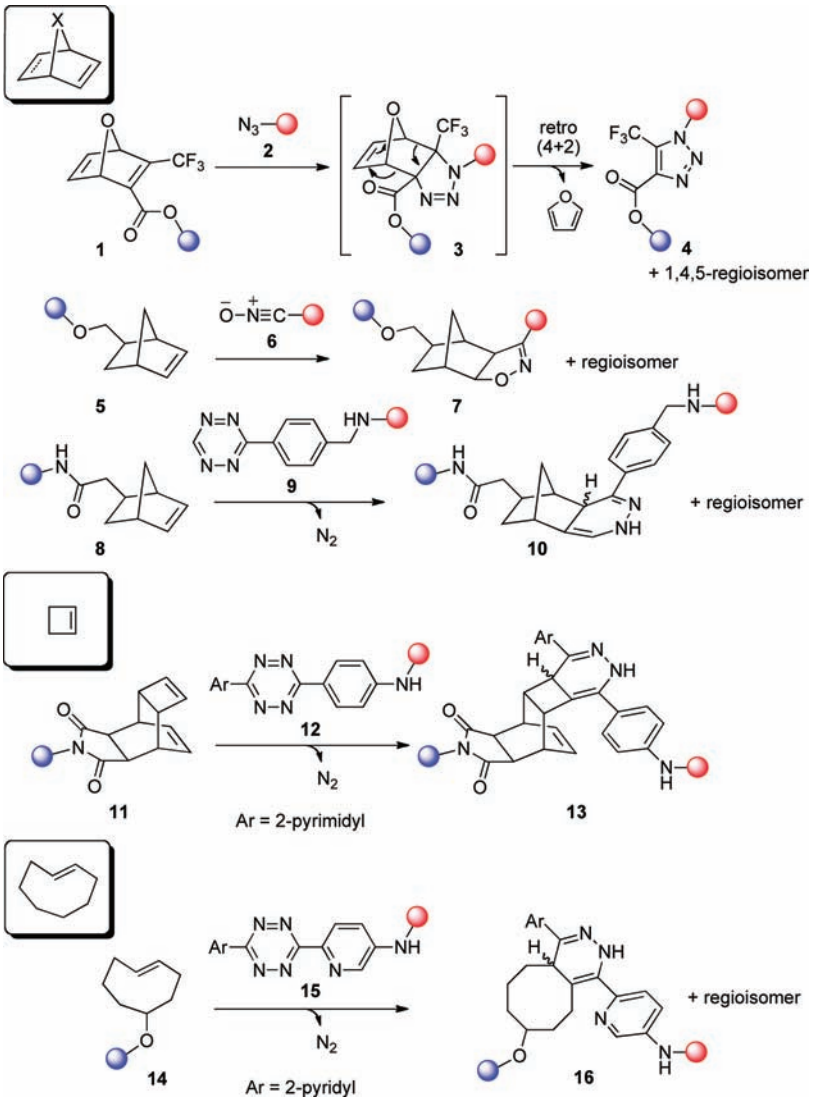


FIGURE 2. Strain-promoted cycloadditions with cyclic alkenes.

Cyclobutene. A range of strained alkenes, apart from norbornene, undergo rate-accelerated cycloadditions. Pipkorn and co-workers[15] constructed a fused cyclobutene-norbornene (**11**) that was reacted with equimolar bis-substituted tetrazine **12**, which led to the desired conjugate **13** in high purity and yield (98%).

***trans*-Cyclooctene.** The most reactive alkene for cycloaddition to tetrazines[14] is *trans*-cyclooctene (TCO, compound **14**). TCO is readily accessible from *cis*-cyclooctene by a variety of synthetic methods or by a photochemical protocol.[16] TCO is the smallest *trans*-cycloalkene that can be isolated in pure form[17] and is highly suitable for a range of HOMO alkene-controlled cycloaddition reactions, due to severe twisting in the crown conformation.[18] Fox et al. were the first to explore TCO cycloaddition with tetrazine for bioconjugation purposes.[19] Via a (4 + 2) cycloaddition of **14** to **15** and subsequent dinitrogen elimination, dihydropyridazine conjugate **16** was formed with an exceptionally high reaction rate constant of 2000 M^{-1} s^{-1} in a water–methanol mixture (9:1), while others reported an even higher rate constant (13 000 M^{-1} s^{-1}) in phosphate buffered saline (PBS) at 37 °C.[20] Such excellent reaction rate constants are unparalleled by any other bioorthogonal reaction pair described in this Account. The resulting dihydropyridazines are stable compounds, although aromatization to the corresponding pyridazines has been observed for compound **13** with Ar = phenyl.[19] Nevertheless, such aromatization does not negatively affect bioconjugation.

Bioorthogonal Ligations with Strained Alkenes. One of the first applications of strained alkenes for bioorthogonal ligation was reported by us in 2007,[11] when we described the reaction of oxanorbornadienes with azides via a tandem

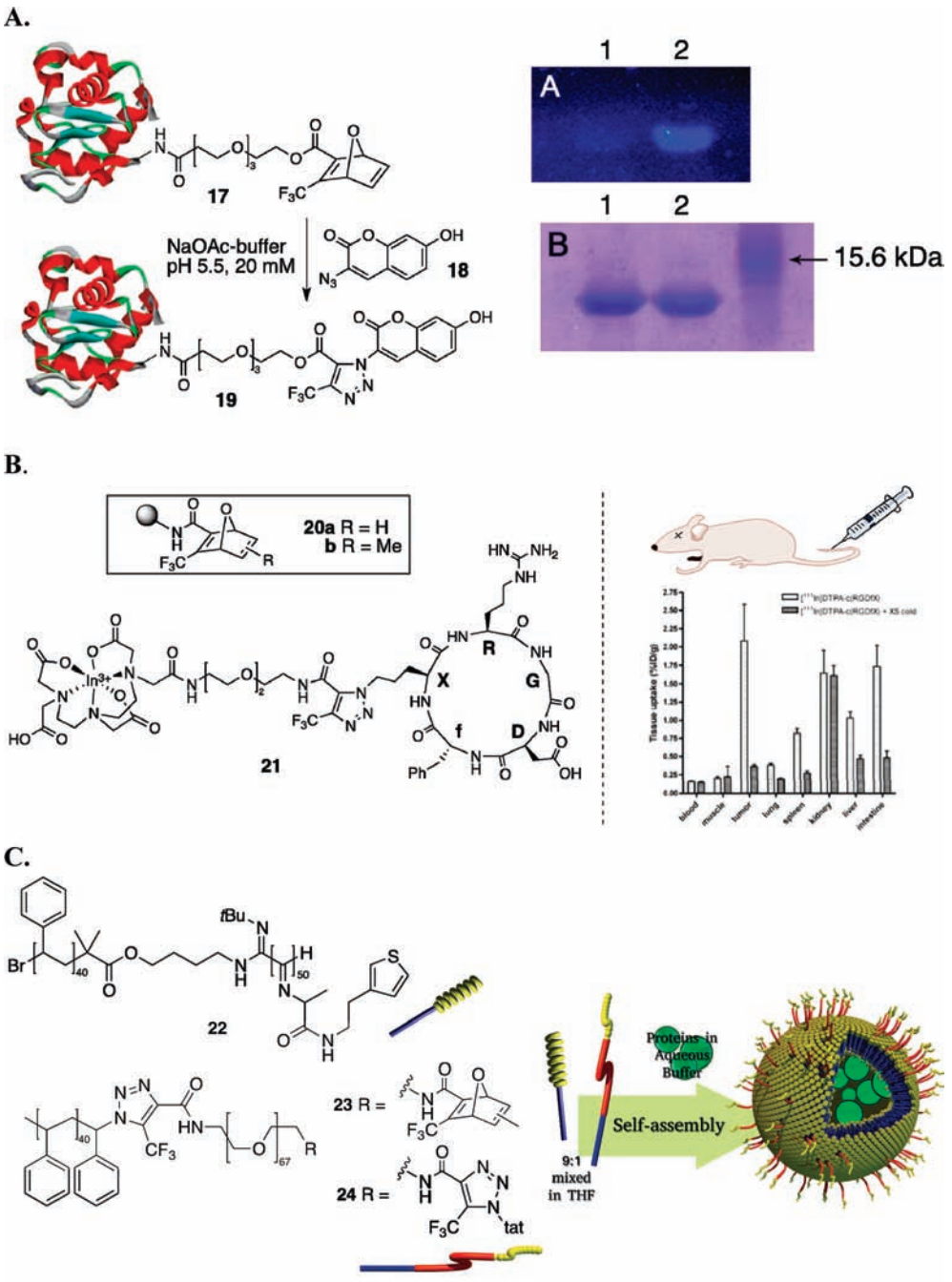


FIGURE 3. Selected bioorthogonal applications of oxanorbornadienes. (A) Fluorogenic reaction on oxanorbornadiene-labeled hen egg white lysozyme.[11] (B) DTPA-RGD conjugate **21** is prepared from **20a** or **20b** and used for scintigraphic imaging of tumors in mice.[24] (C) Construction of polymersome nanoreactors by mixing polymer **22** with either PS-PEG **23** or tat-labeled PS-PEG conjugate **24**.

cycloaddition–retro-Diels–Alder (crDA) reaction.[21] The usefulness of crDA technology for protein modification was demonstrated by subjecting the oxanorbornadiene conjugate **17** of hen egg white lysozyme (HEWL) (Figure 3A), obtained by global acylation, to 7-azido-3-hydroxycoumarin **18**, a known fluorogenic compound.[22] Gratifyingly, upon exposure of the SDS PAGE gel to UV light (λ = 366 nm), a distinct fluorescent band was observed for product **19** on SDS-PAGE (lane 2), while no fluorescence was observed in the case of a control experiment (lane 1, i.e., native HEWL with **18**).

A slight disadvantage associated with unsubstituted oxanorbornadienes (type **20a**, Figure 3B) is that 3–16% competitive addition takes place at the other, electron-rich double bond. To suppress this undesired cycloaddition, we

explored the usefulness of enhanced steric shielding.[23] Indeed, a single methyl group improved the double bond selectivity from 84:16 to 97:3. In a subsequent investigation, amide **20b** was successfully employed in the preparation of DTPA-RGD conjugate **21** from an azido-containing, cyclic RGD-peptide. In fact, tandem crDA to **21** also proceeded effectively in human serum and even in blood, although in blood several unidentified side products were also detected.[24] In addition, we determined the affinity of c(RGD)-DTPA conjugate **21** for $\alpha_v\beta_3$ integrins, heterodimeric cell-surface receptors fundamental to invasion and formation of tumor-induced angiogenesis and metastasis. In a solid-phase binding assay, compound **21** showed an IC_{50} value close to a reference compound (191 nM), which stimulated us to study in detail the usefulness of **21** for in vivo targeting of $\alpha_v\beta_3$-expressing tumors in nude BALB/c mice with subcutaneous SK-RC-52 tumors. Biodistribution studies 2 h p.i. showed a specific $\alpha_v\beta_3$-mediated uptake of [^{111}In]DTPA-c(RGDfX) in tumor and other $\alpha_v\beta_3$-expressing tissues, which shows the potential of crDA for the synthesis of peptide-based radiotracers.

A third example of the crDA reaction for peptide conjugation involves the preparation of a polymersome nanoreactor for the design of functional, artificial organelles.[25] Polymersomes are nanometer-sized, self-assembled vesicles consisting of block copolymers, with presumed suitability for in vivo use. Therefore, PS-PEG-tat conjugate **24** was prepared by crDA of the cell-penetrating peptide tat and PS-PEG-oxanorbornadiene **23**, in turn prepared by crDA of azido-capped polystyrene with a PEG-linked oxanorbornadiene dimer (Figure 3C). Finally, polymersomes were assembled from conjugate **24** and PS-PIAT **22** (1:9 ratio) which where preloaded with GFP prior to cellular incubation. Confocal microscopy showed macropinocytosis-mediated uptake of tat-containing polymersomes (**24**), but not of a control polymersomes. In addition, the specific delivery of enzymes into cells with phagocytic activity was explored by delivering horseradish peroxidase (HRP) into HeLa cells using polymersomes **24**.

Protein and peptide modifications based on strained alkenes are clearly not limited to the crDA reaction. For example, Hilderbrand et al. applied norbornene–tetrazine ligation for cellular imaging by in vivo pretargeting of Her2/neu receptors on human breast cancer cells. In serum, tetrazines conjugated to a near-infrared fluorophore selectively and rapidly labeled a norbornene-modified, pretargeted monoclonal antibody.[13] Simultaneously, it was shown by Robillard et al.[20] that inverse electron-demand

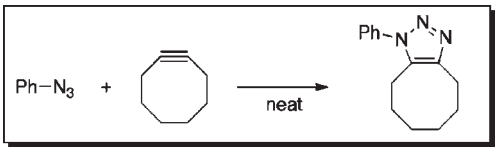


FIGURE 4. Prototypical cyclooctyne reaction with phenyl azide.

Diels–Alder reactions with *trans*-cyclooctene (TCO) instead of norbornene could even be performed in living animals. Thus, reaction between a tumor-localized antibody carrying TCO and ^{111}In-labeled tetrazine proceeded with 52% efficiency in living mice. Noninvasive imaging showed pronounced radioactivity localization in the tumor. The excellent rate and yield of TCO-tetrazine ligation was also utilized in the labeling of a model protein thioredoxin,[19] in a fluorogenic reaction,[26] and for ^{18}F-scintigraphic imaging.[27]

Although significantly slower, cyclobutene-tetrazine conjugation was applied by Pipkorn et al. to conjugate a transporter peptide to the anticancer drug temolozomide, achieving high levels of apototic prostate cancer cells in vitro.[15] Norbornenes have also been incorporated into oligonucleotides for fast reaction with nitrile oxides[12] or tetrazines.[28] The latter norbornene-tetrazine ligation has successfully been applied for the labeling of quantum dots.[29]

Strained Alkynes

Although the reaction rate of TCO-tetrazines cycloaddition (2000 M^{-1} s^{-1} and up) is truly unrivaled, high kinetics are not the only requisite for bioorthogonal application. A drawback of bispyridyltetrazine **15**, for example, is its poor stability in PBS, serum, and blood, with half-lives estimated at 24, 7, and 3 h, respectively, based on reported stability data.[20] Alternative functionalities for reaction with TCO, for example, azides or azoxy compounds, are unsuitable for conjugation purposes due to rearrangement–dissociation of the initial cycloadducts.[18,30]

Another class of compounds known to undergo facile cycloadditions is formed by the cyclic alkynes. Due to severe deformation from the ideal 180°, the triple bond in cycloalkynes having less than nine ring atoms displays high reactivity with dienes, 1,3-dipoles, and other systems.[31,32]

Cyclooctyne. Cyclooctyne is the smallest isolable cyclic alkyne. Cycloalkynes of smaller rings have been generated, but can only be detected by fast spectroscopic means or by indirect trapping. Cyclononynes and larger alkynes are perfectly stable, but show only moderate reactivity in cycloadditions. The reactivity of cyclooctyne was first recognized in 1953 by Blomquist and Liu,[33] whom found that cyclooctyne,

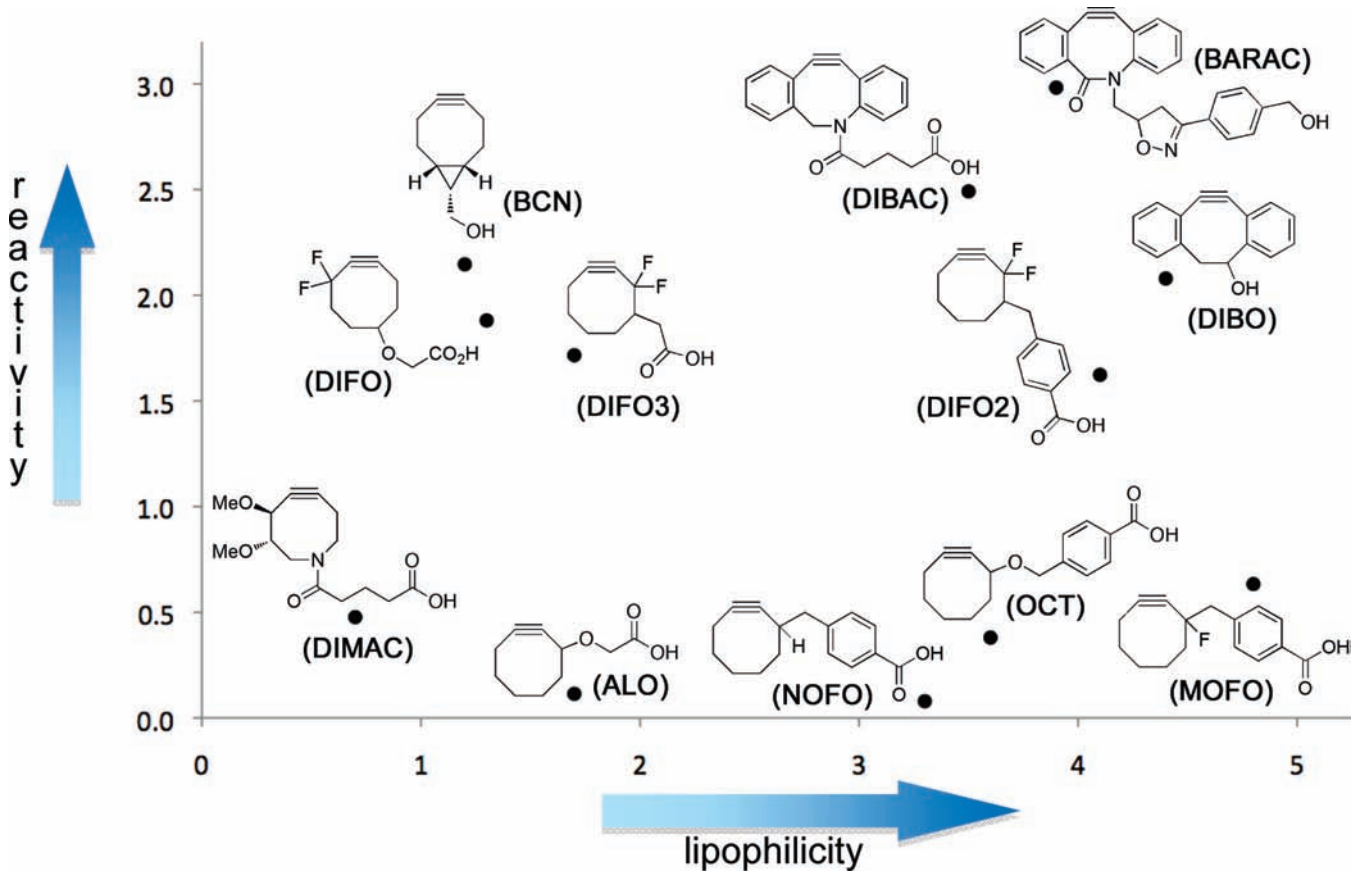


FIGURE 5. Reactivity (with benzyl azide) versus lipophilicity of functionalized cyclooctynes, based on numbers listed in Table 1.

prepared by oxidative decomposition of cyclooctane-1,2-dione dihydrazone, underwent an explosive reaction with (neat) phenylazide (Figure 4). The structure of the resulting (3 + 2) cycloadduct 1-phenyl-4,5,6,7,8,9-hexahydro-1H-cycloocta[d]-[1,2,3]triazole was later corroborated by Wittig and Krebs.[34]

Cyclooctynes for Bioconjugation by Cycloaddition with Azide (SPAAC). It was Bertozzi and co-workers who recognized the potential of cyclooctyne for bioconjugation.[35] At a time when the copper-catalyzed azide−alkyne cycloaddition (CuAAC)[36] was rapidly gaining popularity,[37] it also became apparent that the toxicity of copper(I) often obviates application of CuAAC in living systems.[38] In this respect, strain-promoted alkyne−azide cycloaddition (SPAAC) with cyclooctynes is ideal for bioconjugation since no additional reagents are required. However, reaction rate constants of regular cyclooctynes with azides did not surpass those of the Staudinger ligation ($1.2-2.4 \times 10^{-4}$ M^{-1} s^{-1}).[39] Fortunately, cyclooctyne reactivity can be greatly enhanced by structural modification, for example, by fluorination,[40] by sp^2-hybridization of ring-atoms,[41] or by fusion to cyclopropane.[42] It goes beyond the scope of this Account to discuss in detail all cyclooctyne variants that have been applied in bioconjugation (Figure 5). This topic has been covered by several excellent recent reviews.[43,44] Nevertheless, some general comments on cyclooctynes applied in bioconjugation can be made.

For a reaction to be suitable for bioconjugation, two basic features are of high importance: *reactivity* and *selectivity*. First, high reactivity is clearly a prerequisite for application under highly dilute conditions, for example, in vivo. Second, it is obvious that any bioorthogonal ligation becomes severely thwarted in the case of lipophilic binding to proteins and/or when cross-reactivity with natural biomolecular functionality is operative. For example, lipophilic binding to blood albumins followed by irreversible covalent attachment was held responsible for the large difference between in vitro and in vivo characteristics of a range of cyclooctynes.[45] During in vitro control experiments, we also experienced that covalent reaction with thiol-containing proteins may occur,[46] in analogy with the reported half-life (24 h) for BARAC in the presence of 5 mM glutathione.[41d] Relevant data on lipophilicity (^{c}logP) and experimental reaction rate ($-\log[k \times 10^3]$) are collected in Table 1 and plotted in Figure 5. From these data, it is clear that the most reactive cyclooctyne reported to date is benzofused lactam BARAC

TABLE 1. Lipophilicity and Reactivity of Functionalized Cyclooctynes

entry	name[a]	^{c}logP[b]	k ($\times 10^{-3}$ M^{-1} s^{-1})	$-\log(k \times 10^{3})$	solvent	ref
1	MOFO	4.8	4.3	0.6	CD_3CN	39
2	DIBO	4.4	120[c]	2.1	CD_3CN:D_2O (3:1)	41a
3	DIFO2	4.1	42	1.6	CD_3CN	40b
4	BARAC	3.9	960	3.0	CD_3CN	41d
5	OCT	3.6	2.4	0.4	CD_3CN	35
6	DIBAC	3.5	310	2.5	CD_3OD	41b
7	NOFO	3.3	1.2	0.1	CD_3CN	39
8	cOctOH[d]	1.8	unknown			
9	DIFO3	1.7	52	1.7	CD_3CN	40b
10	ALO	1.7	1.3	0.1	CD_3CN	39
11	DIFO	1.3	76	1.9	CD_3CN	40a
12	BCN	1.2	140	2.1	CD_3CN:D_2O (3:1)	42b
13	DIMAC	0.8	3	0.5	CD_3CN	47

[a]MOFO = monofluorinated cyclooctyne, DIBO = dibenzocyclooctyne, DIFO = difluorocyclooctyne (1st, 2nd, 3rd generation), BARAC = biarylazacyclooctynone, OCT = cyclooctyne (1st generation), DIBAC = dibenzoazacyclooctyne, NOFO = nonfluorocyclooctyne, ALO = arylless cyclooctyne, BCN = bicyclononyne, DIMAC = dimethoxyazacyclooctyne. [b]Calculated by BioByte (embedded in ChemBioDraw 12.0) for the *N*-methylamide derivative of carboxylic acids and the *N*-methyl carbamate derivative of alcohols. [c]See ref **48**. [d]Reaction rate constants not known.

(entry 4).[41d] Rate enhancement by benzofusion was earlier demonstrated by Boons et al. in the development of DIBO (entry 2),[41a] while introduction of an amide nitrogen in the ring, as in DIBAC (entry 6), sprouted from our own laboratory.[41b] The order of reactivity of DIBO < DIBAC < BARAC, that is, 0.17, 0.36, and 0.96 M^{-1} s^{-1}, respectively, is suggestive of a direct correlation between the number of sp^2-hybridized atoms and reactivity.

A drawback of aromatic rings is their lipophilicity. Lipophilic compounds are not only poorly water-soluble but, more importantly, may engage in hydrophobic interactions with proteins. Figure 5 shows ^{c}logP values for aryl-containing cyclooctynes (entries 2, 4, and 6), in the range 3.3−4.8, while saturated systems (entries 1, 3, 5, and 9−13) have lipophilicity constants between 0.8 and 1.7. DIMAC (entry 13)[47] has the lowest ^{c}logP (0.8) but nevertheless has not been broadly applied due to its lengthy synthesis (11 steps, 5% overall yield) and low reactivity. In fact, synthetic (or commercial) *accessibility* may be regarded as the third essential factor that determines the practical value of a particular cyclooctyne. For example, a poor-yielding synthesis has blocked full flourishing of the first cyclooctyne with suitable reactivity, that is, DIFO (entry 11). Second generation DIFOs (entries 3 and 9) are more easily accessible (8−10 steps, overall yields ~25%), while synthesis of DIBAC proved more straightforward and high yielding (9 steps, 40+% overall yield). To date, the most readily accessible cyclooctyne variant is BCN (entry 12), synthesized in four straightforward steps (~50% combined yield of *endo* and *exo* diastereomer).[42b]

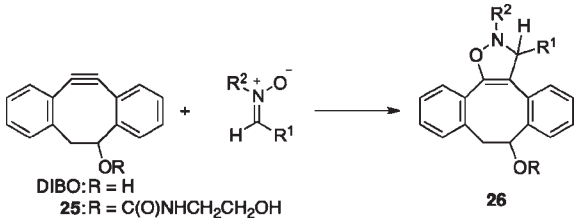
Cycloadditions of Cyclooctyne with Other Dipoles than Azide. Until 2009, the use of cyclooctynes for bioconjugation was focused solely on cycloadditions with azide.[44] However, compared to the reactivity of TCO with tetrazine, the reactivity of SPAAC is very low. Instead of further increasing cyclooctyne reactivity, we were inspired to exploit the promiscuous nature of cyclooctyne in alternative directions, based on the fact that reactions have been reported with carbenes, ketenes, nitrile oxides, diazo compounds, dienes, and others.[31,32]

In a collaborative effort with Boons et al., we reported[48] that benzofused cyclooctynes react rapidly with nitrones (Table 2).[49] The resulting isoxazolines **26** were formed with reaction rates up to 32 times faster than those typical for SPAAC (with benzyl azide), depending on the nitrone substituents. For the more water-soluble DIBO derivative **25**, reaction rates were even 100 times faster upon increasing the water content of the solvent mixture.

Cyclooctyne also smoothly reacts with nitrile oxides.[50] We discovered that nitrile oxides can be instantaneously and quantitatively generated from oximes upon treatment with phenyliodine bis(trifluoroacetate) in a MeOH/water mixture (Figure 6) and reacted with BCN cleanly and rapidly.[51] The expected isoxazoles were formed in only 2−5 min (at a concentration of 0.1 M), with calculated reaction rate constants approximately 10 times faster than SPAAC. A similar report on strain-promoted alkyne−nitrile oxide cycloadditions was reported by Boons et al.,[52] who also disclosed the (significantly slower) reaction of DIBO with diazo compounds.

Bioconjugations and Bioorthogonal Ligations. After its first report as a tool in chemical biology, the cyclooctyne−azide cycloaddition has been applied extensively for chemical reporter strategy, protein modifications, proteomics, and others.[53] We first appreciated the potential of SPAAC for the selective functionalization of azido-containing proteins expressed in auxotrophic bacteria.[54] To this end,

TABLE 2. Reaction Rates of Strain-Promoted Alkyneazide Cycloaddition (SPANC)



starting material	R^1	R^2	solvent (CH_3CN/H_2O)	relative reaction rate (M^{-1} s^{-1})[a]
DIBO	Ph	Me	3:1	0.11
	CH_2CH_2Ph	Me		0.27
	Ph	Ph		1.7
	CO_2Et	Me		32
25	C(O)NHBn	Me	1:1	18
			1:3	46
			1:9	107

[a]Compared to reaction with BnN_3 (SPAAC).

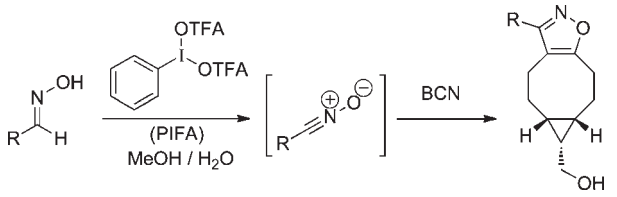


FIGURE 6. In situ generation of nitrile oxide and cycloaddition to BCN.

Candida antartica lipase B (CAL-B) containing five azido groups was treated with DIBAC-PEG_{2000} conjugate **27** or DIBO-PEG_{2000} conjugate **28** in PBS at 30 μM.[41b] Figure 7A clearly shows that labeling with 5 equiv of DIBO-**28** led to a mono-PEGylated product, indicative of reaction with the most accessible azide in the protein only. In contrast, mono-PEGylation already was the main event with just 2 equiv of DIBAC-**27**, while 5 equiv of DIBAC resulted in complete double PEGylated protein. In another paper, we applied BCN for fluorescent staining of cowpea chlorotic mottle virus (CCMV) and for ligation to azido-containing cell surface glycans on MV3-melanoma cells.[42b] Subsequent strepatavidin staining revealed fine, subcellular details, which can discriminate surface glycan distribution states on individual living cells (Figure 7B). Moreover, we followed the migration of the highly invasive MV3 melanoma cells in time, thereby showing the redistribution and accumulation of sialic acid at actin-rich contact sites with collagen fibers, consistent with their role in cell adhesion and migration.

The selective introduction of azides into proteins with auxotrophic bacteria,[54] by genetic engineering,[55] or by chemical azido transfer on proteins,[56] can be highly laborious or

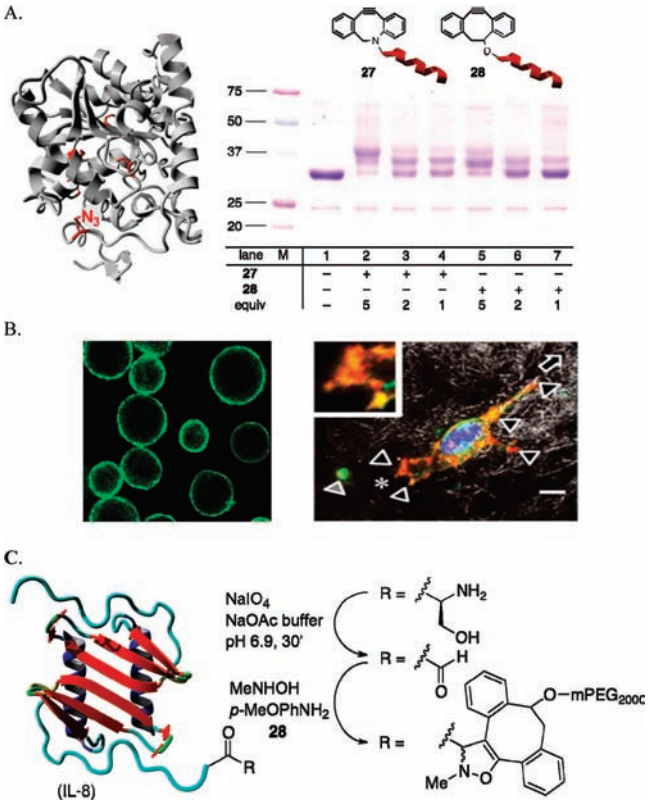


FIGURE 7. Selected bioorthogonal applications of cyclooctynes. (A) PEGylation of CalB containing five azido groups, with DIBAC-PEG **27** or DIBO-PEG **28**.[41b] (B) Fluorescence staining of cell surface glycans on MV3 cells.[42b] (C) N-terminal labeling of IL-8 by SPANC.[48]

inappropriate for single-site protein modification. We realized that chemical *N*-terminal oxidation[57] of proteins leads to the selective introduction of one aldehyde or ketone, a strategy

also applied in oxime ligation.[58] However, because oxime formation is potentially reversible, in particular at acidic pH,[59] we explored whether the intermediate aldehydes could be converted in situ into nitrones for SPANC reaction. A one-pot, three-step protocol was developed for selective *N*-terminal modification of proteins, involving periodate cleavage, nitrone formation, and SPANC, as exemplified for cytokine IL-8 (Figure 7C).[48]

Finally, the strain-promoted cycloaddition of cyclooctynes with nitrile oxides may also be of value for conjugation of peptides and oligonucleotides. We recently showed that in situ generated nitrile oxides react smoothly but slowly with terminal alkynes, whereas reaction is significantly enhanced with cyclooctynes.[51] Finally, it has also been shown that cyclooctynes can be converted into phosphoramidites and incorporated into oligonucleotides for strain-promoted reaction with nitrile oxides[60] or azides.[61]

Outlook

Strain-promoted cycloadditions of cyclic alkenes and alkynes have now established themselves as powerful tools in (reagent-free) bioconjugations.[11,53] The choice for a particular ligation strategy suitable for a specific application is based on different factors. Application of the relatively slow oxanorbonadienes, for example, will most likely be limited to carefully controlled in vitro reactions with azido-containing biomolecules[62] or for fluorogenic labeling of proteins containing free thiols.[63] Compared to azides, norbornenes react with nitrile oxides or tetrazines much faster, but in this case reaction partners are inherently unstable (nitrile oxide) or degrade in the presence of biological functionalities (tetrazine). Nevertheless, tetrazines have enormous potential for bioorthogonal ligation due to the extremely fast reaction with *trans*-cyclooctene, a stable, relatively small, nonlipophilic, and inert functionality. Although at present TCO is not commercially available, wide bioorthogonal application of TCO may be foreseen, including in vivo, in particular if more biocompatible reaction partners are discovered.

A similar bright future lies ahead for cyclooctynes. Although cross-reactivity of the more reactive cyclooctynes with thiols excludes full bioorthogonality, cycloadditions are possible with a much broader range of (small) dipoles, that is, azides, nitrones, nitrile oxides, and diazo compounds. Furthermore, synthetic access to cyclooctynes can be straightforward, and several cyclooctynes have become commercially available.[64] Cyclooctynes are now also finding their way into materials science, for example, for microarrays,[41c] quantum dots,[65] hydrogels,[66] polymers,[62,67] and dendrimers.[68] Finally, it has been shown that temporary masking as a cyclopropene precursor allows for the temporal and spatial generation of cyclooctyne upon subjection to UV-light (355 nM).[69]

Clearly, the potential of metal-free ligations is tremendous, in chemical biology and beyond. Exciting times lie ahead of us to experience where strain-promoted cycloadditions will take us.

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FOOTNOTES

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