Bioconjugate Chemistry

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Strain-Promoted Oxidation-Controlled Cyclooctyne−1,2-Quinone Cycloaddition (SPOCQ) for Fast and Activatable Protein Conjugation

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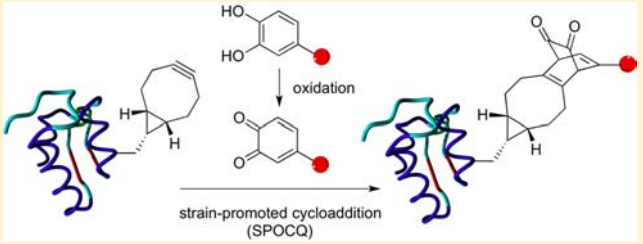
Supporting Information

ABSTRACT: A main challenge in the area of bioconjugation is to devise reactions that are both activatable and fast. Here, we introduce a temporally controlled reaction between cyclooctynes and 1,2-quinones, induced by facile oxidation of 1,2-catechols. This so-called strain-promoted oxidation-controlled cyclooctyne−1,2-quinone cycloaddition (SPOCQ) shows a remarkably high reaction rate when performed with bicyclononyne (BCN), outcompeting the well-known cycloaddition of azides and BCN by 3 orders of magnitude, thereby allowing a new level of orthogonality in protein conjugation.



INTRODUCTION

The modification of proteins is key to many (bio)technological and diagnostic applications, including surface immobilization and conjugation of proteins to specific reporter molecules such as fluorophores, radioisotope labels, or chelators. A well-known example of tailoring of protein properties for clinical application involves the covalent attachment of a polyethylene glycol chain (PEGylation) to increase the circulation half-life of a protein.[1] Proteins have also been conjugated to drugs and have been used as drug-delivery systems for therapy or diagnostic purposes.[2,3] In this respect, a high level of control over the site of modification is particularly relevant, since a structural change can have direct impact on protein function. Amide bond formation with lysine side-chains or thiol-maleimide chemistry for the reaction with cysteines generally lack site-selectivity, because either amino acid is typically present multiple times in a given protein. An elegant strategy to enable chemoselective conjugation under mild conditions entails the site-specific generation of non-proteinogenic keto or aldehydo-functionality, followed by oxime ligation.[4−6] Selective protein labeling can also be achieved via azide-chemistry, i.e., Staudinger ligation,[7] Cu(I) catalyzed alkyne−azide cycloaddition (CuAAC),[8,9] or strain-promoted cycloaddition of cyclooctynes (SPAAC).[10−12] Alternative 1,3-dipoles, such as nitrones (SPANC),[13,14] nitrile oxides (SPANOC),[15] diazo compounds, or sydnones have also been found suitable for this purpose.[15,16] Many different technologies have been developed to site-specifically introduce the appropriate chemical handle into a protein, involving genetic, enzymatic, and chemical approaches.[17−19] The strain-promoted inverse electron-demand Diels−Alder cycloaddition (SPIEDAC) of cyclooctynes or *trans*-cyclooctenes with tetrazines has recently been developed as a particularly fast procedure to site-selectively label proteins.[20−23] One shortcoming of the above conjugation strategies is the lack of temporal control of activation.

Two elegant exceptions in this regard include the cycloaddition of alkenes and nitrile imines, generated in situ by UV irradiation (300 nm) of tetrazoles,[24−27] and a phototriggered click reaction upon in situ generation of cyclooctynes from a cyclopropene precursor with 350 nm light.[28] However, short-wavelength conditions may not always be compatible with light-sensitive molecules. Controllable, site-specific reactions are highly valuable for multiplexing of (bio)molecules and expansion of labeling diversity. Early studies during the 1970s and 1980s described the reaction of plain cyclooctyne with various 1,2-quinones in organic solvents.[29−31] It has also been shown that 1,2-quinones can be generated on proteins by selective periodate oxidation of genetically incorporated 3,4-dihydroxy-L-phenylalanine (L-DOPA), leading to slow (5 h) conjugation with a polysaccharide.[32] We aimed to explore the facile catechol-to-quinone conversion as a fast, chemically activatable bioconjugation by strain-promoted chemistry. In this paper, we describe the potential of 1,2-quinone cycloadditions with a highly reactive cyclooctyne variant, i.e., bicyclo[6.1.0]non-4-yne (BCN), as a novel bioconjugation tool. We show that the reaction of 1,2-quinone with BCN is highly suitable for extremely fast and selective in vitro labeling of proteins. Moreover, the cycloaddition can be selectively activated by the oxidation of the catechol moiety to 1,2-quinone providing temporal control over the reaction (Scheme 1).

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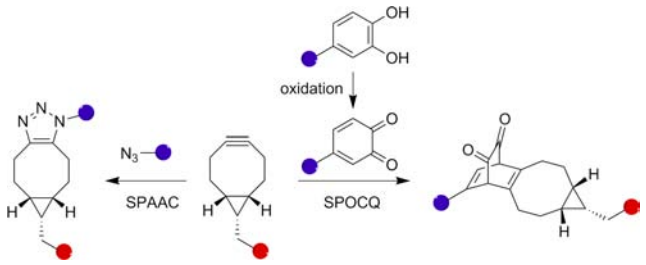
Scheme 1. Strain-Promoted Alkyne-Azide Cycloaddition (SPAAC) with BCN and Strain-Promoted Oxidation-Controlled Cyclooctyne−1,2-Quinone Cycloaddition (SPOCQ)



RESULTS AND DISCUSSION

To assess the scope and limitations of the cyclooctyne−1,2-quinone reaction, first a model system was studied in detail. We selected the monosubstituted 4-*tert*-butyl substituted quinone (**1**) as a (relatively) stable and simple model substrate to investigate the reaction kinetics for cycloaddition with BCN.[33] Hence, 4-*tert*-butyl-1,2-quinone (**1**) was prepared by oxidation of 4-*tert*-butyl-1,2-catechol with sodium periodate and isolated as a pure solid without column chromatography.[34,35] Next, cycloaddition was investigated by adding 1.5 equiv BCN (**2**) to a solution of **1** in methanol/water (1:1) (Figure 1a). As

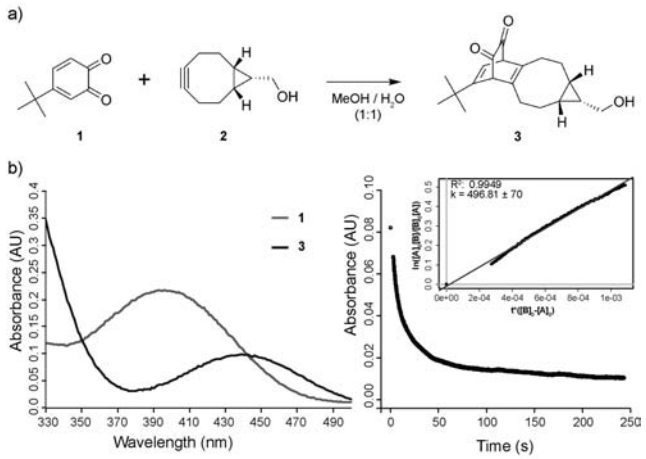


Figure 1. Reaction kinetics of SPOCQ. (a) Cycloaddition of 4-*tert*-butyl-1,2-quinone (**1**) with BCN (**2**). (b) UV−vis spectrum of **1** and SPOCQ product **3** and determination of reaction rate constant in MeOH/H_2O (1:1) at room temperature using decay in absorbance of **1** at 384 nm. Due to the high rate of this reaction, there is a small variation in the time from mixing the reactant until starting the measurement, which causes the deviation at the beginning of the fitting line.

anticipated, a rapid reaction took place as indicated by immediate color change from red to yellow, and the formation of a new product based on TLC analysis. Purification by silica column chromatography led to the isolation of product **3** as a mixture of stereoisomers with a combined isolated yield of 80%, along with a small amount of an aromatic compound, formed by a retro-Diels−Alder reaction resulting in decarbonylation of **3** (see Supporting Information, SI).

The apparent fast reaction of *tert*-butyl-1,2-quinone with BCN stimulated us to investigate in detail the second-order rate reaction constant of this cycloaddition, which we termed strain-promoted oxidation-controlled cyclooctyne−1,2-quinone cycloaddition (SPOCQ). Initial attempts in this respect were focused on monitoring the reaction progress with NMR, by integrating the disappearance/formation of the representative peaks. However, the reaction was too fast to monitor; i.e., (near) complete conversion was already observed at the first time point. Therefore, we turned our attention to UV-spectroscopy as a potential tool for measuring reaction conversion, based on the assumption that the highly conjugated 1,2-quinone system would display much enhanced absorbance with respect to the nonconjugated product **3**.[28] Indeed, UV-spectroscopy of the respective components indicated (Figure 1b) a large difference in absorbance between **1** and **3**, most notably at 384 nm. We therefore monitored the (exponential) decay of the absorbance of **1** at 384 nm over time upon reaction with BCN in 1:1 methanol/water (Figures 1 and S1 (SI)). Based on these measurements, the reaction rate constant for SPOCQ was calculated to be 496 ± 70 M^{-1} s^{-1}, roughly the same order of magnitude as the reaction of BCN with tetrazines (400−1200 M^{-1} s^{-1})[23] and the reaction of spirocyclopropene with nitrile imines (890 M^{-1} s^{-1}),[25] but orders of magnitude faster than strain-promoted cycloaddition of azides and cyclooctynes (typical values 0.01−1 M^{-1} s^{-1}).[36] Furthermore, the cycloaddition of **1** with BCN outperforms the SPANC reaction (highest values up to 39 M^{-1} s^{-1}) or the recently identified reaction of BCN with sulfenic acid (12 M^{-1} s^{-1}).[13,37] Taken together, only the SPIEDAC reaction of tetrazine with trans-cylcooctene shows a significantly shows a significantly higher reaction rate constant (>5000 M^{-1} s^{-1}) than SPOCQ.[23] We also investigated the cycloaddition of **1** with cyclooctyne derivative dibenzoazacyclooctyne (DIBAC, also known as DBCO), previously developed by us as well as by others.[38,39] While DIBAC is known to react faster with (aliphatic) azides than BCN, surprisingly, the reaction rate constant for cycloaddition with 1,2-quinone was found to be 1000× slower (0.19 M^{-1} s^{-1}) than that for reaction with BCN, as determined by NMR, and is comparable to the rate constants for previously described SPAAC reactions (Figure S2 (SI)). The latter finding is in line with the observation that 1,2,4,6-tetrazines, another type of electron-deficient diene-reagent, as well as electron-poor aromatic azides,[40] react quickly with BCN, but not with DIBAC.[41,42]

Next, we explored the feasibility of SPOCQ for bioconjugation. First, we turned our attention to the selective labeling of peptides containing a catechol moiety. Model peptide **4**, readily generated and purified by standard (solid phase) peptide synthesis, was first oxidized for 30 min with sodium periodate (1 equiv) to generate the corresponding 1,2-quinone in situ, followed by incubation with BCN-biotin (**5**). The progress of reaction was analyzed by high performance liquid chromatography (HPLC) using a reverse phase C-18 column. A new peak was identified with a retention time of 9 min (Figure S3 (SI)). Mass spectrometry analysis of the newly formed product revealed a corresponding mass matching with that of the anticipated cycloadduct product **6** (see SI). When peptide **4** was not oxidized prior to incubation with **5**, no reaction product was detected (Figure S3 (SI)). These results show that the catechol moiety is inert toward BCN and can be controllably activated by oxidation to undergo reaction with BCN. The large difference in reaction rate constants of BCN with azide and 1,2-quinone triggered us to further investigate whether mutually orthogonal labeling could be achieved by the combination of SPOCQ and SPAAC. Thus, a competition

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experiment was designed with peptide **4** and N-terminally azide-functionalized peptide **7**. HPLC and MS analysis of the reaction of oxidized peptide **4** with **5** and **7** revealed the formation of the cycloaddition product of **4** and **5**, whereas the mass of the product from the SPAAC reaction between **5** and **7** (anticipated product **8**) was not detected (Figures 2 and S4, (SI)). However, when we repeated the experiment without adding the oxidized peptide, mass spectrometry confirmed the presence of the expected SPAAC product (Figures 2 and S4 (SI)). These findings show that the higher reaction rate of SPOCQ outcompetes SPAAC and can be used for selective labeling. Moreover, since the catechol moiety is inert toward BCN and only reacts after oxidation, it can be used as an activatable handle in conjugation studies.

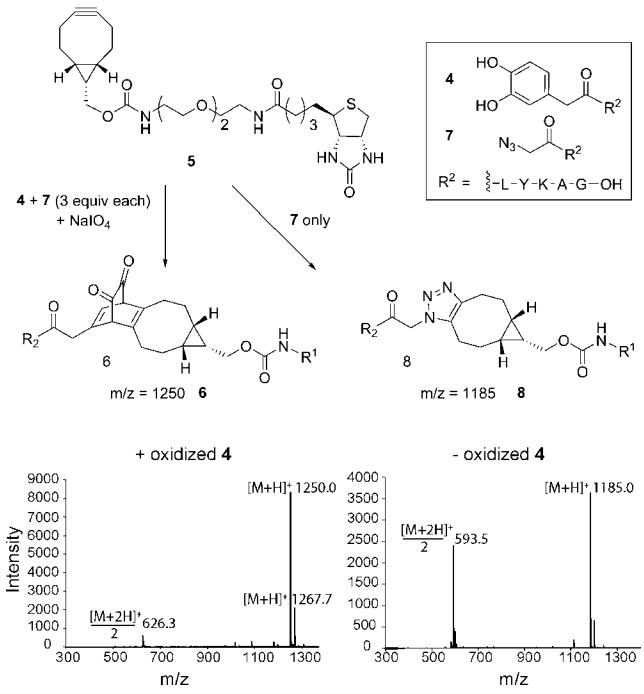

Figure 2. Competition experiment of 1,2-quinone- and azido-functionalized peptide (oxidized **4** and **7**, respectively) with BCN-biotin (**5**). Competition experiment with and without oxidized peptide **4** was analyzed by HPLC followed by ESI-MS. R^1 = PEG_3-(+)-biotin.

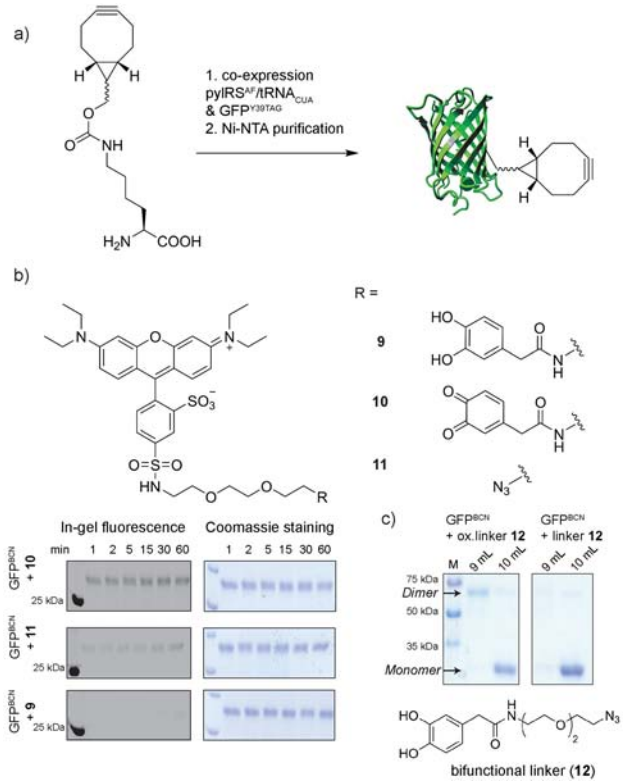

Figure 3. SPOCQ for bioconjugation. (a) Site-specific incorporation of BCN into GFP. (b) In-gel fluorescence assay and corresponding Coomassie stained protein gels of fluorescently labeled GFP^{BCN} using SPOCQ and SPAAC. (c) Protein gel showing controlled dimer formation of GFP^{BCN} using bifunctional linker **12** after oxidation. Dimers were separated from monomers using size exclusion chromatography (full chromatogram in SI). Elution fractions containing dimer and monomer are indicated.

With these exciting findings in hand, we speculated that it should be possible to use SPOCQ for protein labeling. Although it is known that 1,2-quinones react with naturally occurring functionalities such as amines and thiols, chemoselective labeling with SPOCQ may still be feasible, since the reaction of BCN with 1,2-quinone displays such a high reaction rate.[32,43−45] For protein labeling purposes, BCN can be readily incorporated into proteins via genetic code expansion as has been described earlier.[21,23] Thus, we used a C-terminally His_6-tagged green fluorescent protein (GFP^{BCN}) that carried an amber codon (TAG) mutation at position 39 as a model protein and genetically encoded BCN-lysine using a previously engineered version of the pyrrolysine tRNA-synthetase (pylRS) from *Methanosarcina mazei* and its corresponding suppressor $tRNA_{CUA}$ (Figure 3a).[12] After recombinant expression, GFP^{BCN} was purified by means of Ni-NTA chromatography. A catechol-containing fluorescent dye (**9**), synthesized in three steps from sulforhodamine B acid chloride and dihydroxyphenyl acetic acid (see SI), was then oxidized (**10**) and subsequently incubated (4 equiv) with GFP^{BCN} at room temperature for 1 h (Figure 3b). Wild-type GFP (GFP^{WT}), that contains a tyrosine residue at position 39, was used as a negative control. Gratifyingly, in-gel fluorescence analysis revealed that GFP^{BCN} selectively reacts with **10** (Figure S5 (SI)). Mass spectrometry analysis further confirmed the formation of a single product peak even when the oxidizing agent was directly added to a mixture containing GFP^{BCN} and **9** (Figure S6 (SI)). No ligation product was observed in the absence of oxidizing agent (Figure S6 (SI)). Based on mass spectrometry analysis, quantitative labeling was observed with small peptides (Figure S7 (SI)) and labeling efficiency of proteins was estimated to be 90%. Moreover, coincubation of GFP^{BCN} with bovine serum albumin and **10** showed that reaction with GFP^{BCN} was much faster than the reaction of other nucleophiles such as amines or thiols present in proteins, which limits undesired nonspecific labeling with lysine or free cysteine (Figure S8 (SI)). If cross-reactivity becomes a problem for future in vitro applications, thiols can potentially be capped using sulfhydryl-reactive chemical groups such as iodoacetamide or maleimide.

Next, we followed the reaction of GFP^{BCN} with **10** as a function of time to estimate whether the high reaction rate of SPOCQ, observed for small molecules, also translates into fast labeling of proteins. Samples were taken at various time points and subsequently quenched with an excess of BCN alcohol. We also performed the experiment with an azido-dye (**11**) to directly compare the labeling kinetics of 1,2-quinone cyclo-

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addition to that of SPAAC. In-gel fluorescence analysis showed that, already after 1 min, GFP^{BCN} was clearly fluorescently labeled with **10** and reached saturation within a few minutes (Figure 3b). In contrast, only faint labeling of GFP^{BCN} with **11** was observable and did not reach completion within 1 h. To prove that the reaction of **10** with GFP^{BCN} was indeed oxidation-controlled, we incubated GFP^{BCN} with **9** and did not observe any fluorescent labeling (Figure 3b). As expected from previous cycloaddition studies with GFP^{BCN} and absorbance/fluorescence measurements, structural integrity of GFP was not influenced by SPOCQ (Figure S9, SI).[21,23]

As a first attempt toward multiplexing, we sought to use the orthogonality of SPOCQ and SPAAC to form protein dimers. We synthesized a bifunctional linker **12** (Figure 3c) containing both an azide and a catechol moiety. A small excess of the linker (5 equiv), after oxidation with periodate, was then incubated with GFP^{BCN} for 1 h at room temperature. Subsequently, the excess of linker was removed by spin filtration and another equivalent of GFP^{BCN} was added, leading to the desired formation (20−30%) of protein dimer, as analyzed by gel electrophoresis and Coomassie staining (Figure 3c). As expected, no dimer formation was observed without prior oxidation of the linker. Although other studies using bifunctional linkers to form protein dimers reported slightly higher yields (up to 50%),[46,47] we envisage that further optimization of dimer formation could be achieved, e.g., by use of a longer linker or incubation at higher concentrations. Nevertheless, these experiments demonstrate the feasibility of selective activation-conjugation by a combination of SPOCQ and SPAAC for multiplexing. Finally, it must be noted that the formed dimers were readily separated from the unreacted monomers with size exclusion chromatography, yielding a population of well-defined protein dimers (Figure S10 (SI)).

CONCLUSION

We have described a new bioconjugation reaction based on cycloaddition of bicyclo[6.1.0]nonyne and 1,2 benzoquinone derivatives. This new cycloaddition, named SPOCQ (strain-promoted oxidation-controlled cyclooctyne 1,2-quinone cycloaddition) shows an extremely high reaction rate constant, several orders of magnitude faster than for SPAAC. We think that SPOCQ will add a new temporally controlled and fast reaction to the bioconjugation toolbox, since a variety of catechol derivatives as well as BCN-derivatives are readily accessible, and furthermore, both BCN and catechol-bearing amino acids can be genetically incorporated into proteins[48] and periodate treatment is a broadly applied tool for chemoselective oxidation of proteins.[49] Although direct application of SPOCQ as a bioorthogonal tool for in vivo labeling is obviated due to the requirement of periodate, it is not excluded that for future applications, the oxidation of the catechol could also be mediated by an enzyme (e.g., catechol oxidase)[50] providing better compatibility of the latter oxidation step. Research along those lines is currently ongoing in our laboratories.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures, syntheses of all compounds, NMR spectra, LC/MS data, additional kinetic data, and protein data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): F.L.V.D. is CSO of SynAffix.

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