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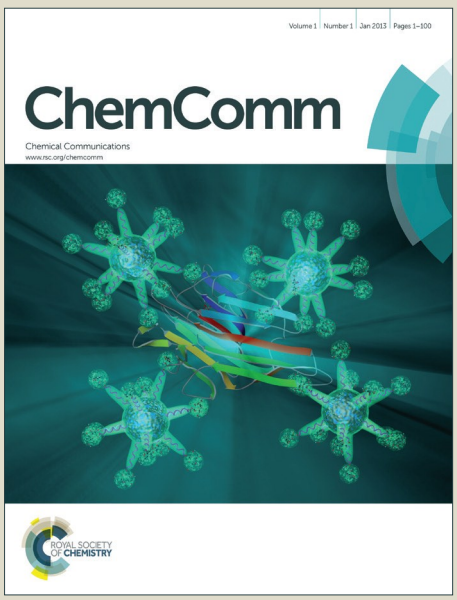

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Study and Application of Noncatalyzed Photoinduced Conjugation of Azides and Cycloocta-1,2,3-selenadiazoles

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The non-catalyzed cycloaddition of eight structurally different azides with cyclooctyne generated in-situ by the photolysis of cycloocta-1,2,3-selenadiazole gives 1,2,3-triazole derivatives as the main products. The application of this reaction was demonstrated on photoconjugation reaction of cycloocta-1,2,3-selenadiazole with an avidin-modified biotin complex to introduce a new strategy in the non-catalyzed synthesis of bioconjugates.

Strain-promoted alkyne-azide cycloaddition (SPAAC) was first described in 1961 by Krebs, who reported the reaction of cyclooctyne with phenylazide affording 1,2,3-triazole as a product.[1] This reaction has been extensively studied as a copper-free conjugation (click) reaction since 2004, when Bertozzi and co-workers used SPAAC as a biorthogonal reaction with biotinylated cyclooctyne for labeling biomolecules and living cells for the first time.[2] Subsequently, various cyclooctyne derivatives and their aza analogues have been designed[3,4] to improve the kinetic parameters of this reaction and used in biological applications, such as cell labeling,[5,6] enzyme screening,[7] fluorescent imaging[8,9] or radiolabeling.[10,11]

The complex multi-step procedures needed for the construction of a triple bond in substituted cyclooctyne systems are the main drawbacks for their applications.[12] On the other hand, the photochemical production of cyclooctyne ring in 5,6-didehydro-11,12-dihydrodibenzo[*a,e*]-cyclopropa[*c*][8]annulen-1-one from 6,7-dihydro-1H-dibenzo[*a,e*]-cyclopropa[*c*][8]annulen-1-one developed by Popik and co-workers[13] and used for cell labeling, is an elegant procedure, but it suffers from a rather difficult synthesis of the starting material.

A cyclooctyne system can also be generated from the corresponding 1,2,3-selenadiazole, which is easily prepared by converting cyclooctanone to its semicarbazone, followed by its subsequent reaction with selenium dioxide.[14-16] However, the transformation of cycloocta-1,2,3-selenadiazole derivatives to cyclooctynes requires harsh conditions, such as thermolysis at temperatures over 115 °C,[17-19] thermolysis on copper powder,[20-24] or a reaction mediated by *n*-BuLi.[14] Such conditions limit the direct use of cycloocta-1,2,3-selenadiazoles for the labeling of biomolecules and the isolation of a generated cyclooctyne system must precede the azide-cyclooctyne conjugation.

Transformation of a 1,2,3-selenadiazole derivative to system bearing triple bond is published only by Krantz and co-workers, who described the formation of ethynylselenol by light-induced decomposition of 1,2,3-selenadiazole[25]. The formation of the product was indicated only by IR spectroscopy in an argon or nitrogen matrix at 8 K. Other examples of the photochemical transformation of 1,2,3-selenadiazole derivatives have also been reported, but photoproducts other than alkynes were formed. For example, the irradiation of 4,6-dihydrofuro[3,4-*d*][1,2,3]selenadiazole gave selenirene and selenoketone derivatives, benzo[*d*][1,2,3]selenadiazole was converted to 6-fulveneselone,[26] simple 1,2,3-selenadiazole afforded etheneselenone,[27] and its 4-ethoxycarbonyl derivative gave the corresponding 1,3-diselenole derivative.[28]

In this work, we studied the photochemically-induced noncatalyzed conjugation reaction of cyclooctyne generated in situ from cycloocta-1,2,3-selenadiazole **1** with structurally different azides **2a-h** at ambient temperature to form 1,2,3-triazoles **3a-3h** (Scheme 1).

The azides **2a-h** represent substrates possessing different electronic and steric properties.

Cycloocta-1,2,3-selenadiazol **1** was prepared from 2-cyclooctylidene-hydrazinecarboxamide according to the previously described procedure.[29] Azides **2a-c**[30] and **2d**[31] were synthesized from the corresponding amines Benzyl azide **2g** was prepared from benzylchloride.[32] 5- and 5´-azides derived from 5-methyluridine were synthesized according to our recently published procedure.[33] The analytical standards, triazoles **3a-h**, were prepared by heating a mixture of

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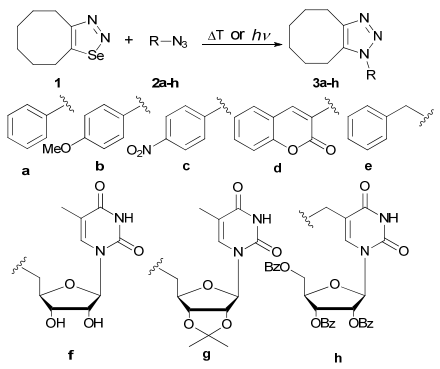
cycloocta-1,2,3-selenadiazole **1** and the corresponding azide **2a-h** (see ESI).



Scheme 1: Noncatalyzed Conjugation Reactions of Cycloocta-1,2,3-selenadiazole **1**.

The irradiation wavelength had to be carefully chosen for each pair of reactants. As the absorption spectrum of the principal chromophore cycloocta-1,2,3-selenadiazole **1** (Figure S4) partially overlaps with those of the azides **2a-2h** (Figures S5–S12) and the photoproducts **3a**–**3h** (Figures S13-S20), the undesired light absorption by the azides should be avoided or at least be largely suppressed. The absorption tail of the starting material **1** allowed us to use irradiation wavelengths up to 325 nm (Figure S4).

The azide **2e** has been selected as a reagent for an initial photochemical reaction study because it shows no absorption above 300 nm (Figure S9). During the irradiation of a solution of equimolar amounts of **1** and **2e** in methanol (c = 3 × 10^{-3} mol dm^{-3}) at 313 nm, a red solid precipitated. According to UV/VIS spectrum, having absorption maximum at λ_{abs} of approximately 500 nm, the solid was identified as selenium nanoparticles with a diameter of approximately 180 nm[34] (Figure S22), which may become an important internal optical filter. Upon exhaustive irradiation, triazole **3e** was found to be the major product formed in a 28% yield, whereas 62% of azide **2e** remained unreacted in the solution according to GC/MS analysis (Figure S23). Due to the thermal instability of the 1,2,3-selenadiazoles,[35] GC/MS could not be used to determine the concentration of the unreacted derivative **1**. Therefore, NMR was used to monitor reaction progress.

Upon irradiation of a solution of **1** (c ~ 0.03 mol dm^{-3}) and **2e** (c ~ 0.03 mol dm^{-3}) in methanol-d_4 in a NMR tube (Pyrex glass; λ > 280 nm, Figure S3) by a medium-pressure Hg lamp for 2 h, the conversion reached 38%, and the triazole **3e** was a major product. The product formation was evident from appearance of the new triplet signals at 2.81 and 2.94 ppm assigned to the methylene group of the triazole derivative and a decreased signal intensity for the methylene groups of 1,2,3-selenadiazole **1** at 3.32 ppm (Figure 1). The ^{1}H NMR spectrum of the product obtained by irradiation corresponds to that of the isolated triazole **3e** prepared by thermolysis (Figure S33).

To detect cyclooctyne as a possible primary photoproduct, irradiation of a solution of **1** in methanol-d_4 (c ~ 0.04 mol dm^{-3}) was performed for 1 h. The ^{1}H NMR showed the signals at 1.64 and 2.10 ppm (Figure S46), what corresponds to the spectrum of cyclooctyne reported in the literature.[36] A new signal in the ^{13}C NMR spectrum at 93.8 ppm and three signals in the range of 20–35 ppm (Figure S47) were also attributed to cyclooctyne in accordance with the published data.[37]

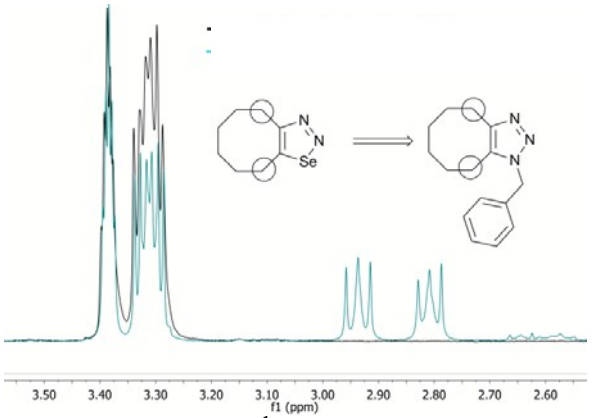


Figure 1: A detail of the ^{1}H NMR spectra of a mixture of 1,2,3-selenadiazole **1** and triazole product **3e** before irradiation (black line) and after 2 h of irradiation (blue line).

The cyclooctyne half-life in the mixture with azide **2e** was estimated to be approximately 40 min (for details see ESI; Figure S1). The quantum yield of triazole **3e** formation upon irradiation of a methanolic solution of the 1,2,3-selenadiazole **1** (c = 1.9 × 10^{-3} mol dm^{-3}) in the presence of **2e** (c = 6.1 × 10^{-3} mol dm^{-3}) at λ_{irr} = 313 nm was determined to be Φ (**3e**) = 0.10 ± 0.05 using valerophenone[38,39] as an actinometer.

To determine the multiplicity of the excited state involved in the cyclooctyne formation, the influence of oxygen as a triplet quencher on the reaction rate was studied. Degassed, aerated and oxygenated solutions of **1** and **2e** in methanol-d_4 were irradiated using a medium-pressure Hg lamp in an NMR tube. The changes in the concentration of **1** at different reaction conversions were followed by ^{1}H NMR (Table S1). The presence of oxygen evidently but not significantly slowed the reaction, thus the productive triplet-excited state lifetime must be relatively short and its reaction competes with a diffusion-limited quenching, or the singlet state is also involved in the reaction.

In addition, sensitization of **1** by triplet-excited benzophenone was performed to find whether the same process occurs in the triplet manifold. Irradiation of a degassed solution of **1**, **2e** and benzophenone in methanol resulted in the formation of **3e** (Table 1, Figure S49); the same product as that obtained by direct irradiation) in substantially higher yields compared to those observed when the same reaction mixture was purged with oxygen. The irradiation wavelength was 366 ± 1 nm to ensure that benzophenone is the only absorbing species (Figure S4 and Figure S21). Table 1 also demonstrates that oxygen quenched the sensitized production of **3e**. Such a result supports further our assumption that **3e** is produced at least in part from triplet-excited **1**.

Table 1. Sensitization of **1**[a]

Irradiation time (h)	Yield of **3e**/%[a]	
	O_2[b]	N_2[c]
2	4	7
16	24	41

[a] **1** (c ~ 0.03 mol dm^{-3}), **2e** (c ~ 0.03 mol dm^{-3}) and benzophenone (c ~ 0.3 mol dm^{-3}) in methanol-d_4 irradiated at 366 nm; the yields were determined by ^{1}H NMR. [b] Sample was

purged with oxygen for 10 min. [c] Sample was purged with nitrogen for 10 min.

The photolysis of an equimolar mixture of **1** and the corresponding azide **2a-d**, **f-h** (c ~ 0.03 mol dm^{-3}) was carried out afterwards, and the reaction conversion was monitored by NMR (see Table S2, Method I). The lowest triazole yield (6-8%) was observed for 3-azidocoumarine **2d**, which has been reported to be photolabile.[43] For the other azides **2a-c** and **2f-h**, triazole yields were 11−39%. Further irradiation did not increase the conversion, which may indicate a role of selenium as an internal filter (see above) and potential side photoreactions of the azides known from literature.[40-42] .

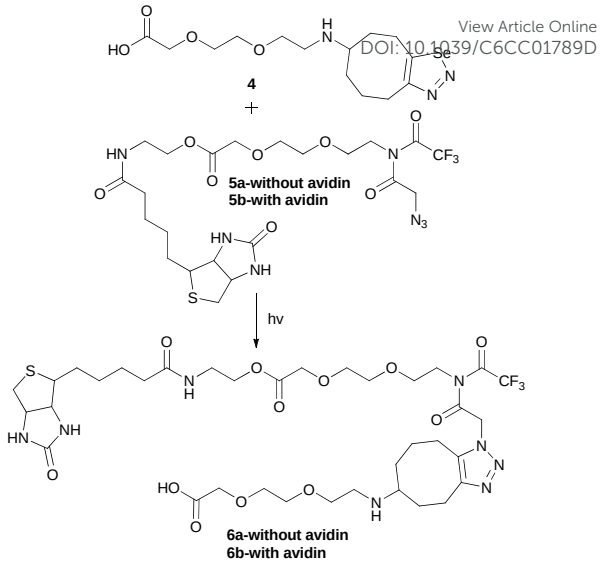
To enhance the reactivity of cycloocta-1,2,3-selenadiazole **1** by avoiding inadvertent photolysis of the azides **2**, a solution of **1** in methanol-d_4 was initially irradiated for 1 h to form cyclooctyne, and then an equimolar amount of the corresponding azide **2** was added. Subsequently, the mixture was kept in the dark, and the reaction conversions were monitored by NMR (Table S2, Method II). Surprisingly, the triazole yields were similar or lower compared to those previously determined. The only exception was the reaction of azide **2d** (the most photoactive azide[43]), where the post-irradiation addition of this azide prevented its photodecomposition and the total yield of triazole increased. In all cases, the starting cycloocta-1,2,3-selenadiazole **1** always remained in the reaction mixture. Because of selenium internal filter formation we were not able to further increase the yield of the triazoles **3** under the conditions used.

To demonstrate the applicability of cycloocta-1,2,3-selenadiazole photoconjugation reaction with azides, labeling of biomolecules was studied. Selenium nanoparticles (Figure S22), which are formed during the reaction, have already been proven to be nontoxic[44], what meets the basic requirements for conjugation reactions applied in molecular biology.

First we prepared a modified derivative of cycloocta-1,2,3-selenadiazole **4** having a PEG moiety for its better water solubility and the azidobiotine **5a** (Scheme 2, the synthesis is described in ESI) as model reaction partners and performed the photoinduced conjugation.

The reaction mixture was irradiated with a medium-pressure mercury arc filtered through the Pyrex glass of an NMR tube for 1 h. The ^{1}H NMR analysis showed the formation of new signals at approximately 5.80 ppm and a decrease in the signal intensity of azidomethylene group in the starting material at approximately 3.42 ppm (Figure 2, S51). The MS spectrum of the product of this reaction also provided evidence for the formation of triazole **6a** (see Figure S53).

The reactivity was then tested on modified protein **5b** as a biomolecule representative. Complex **5b** was prepared by mixing of avidin with modified biotin **5a** (c ~ 0.013 mol dm^{-3}). The concentration of **5a** was higher than corresponds to the binding capacity of avidin to allow monitoring of the reaction course by an NMR analysis of the non-bound biotinylated azide **5a** present in reaction mixture in sufficient concentration. After the addition of 1,2,3-selenadiazole **4**, the reaction mixture was irradiated with a medium-pressure mercury arc filtered through the Pyrex glass of an NMR tube for 1 h. In ^{1}H NMR spectrum we observed the same changes as in reaction without avidin.



Scheme 2: Conjugation reaction of modified cycloocta-1,2,3-selenadiazole and appropriate azido derivatives.

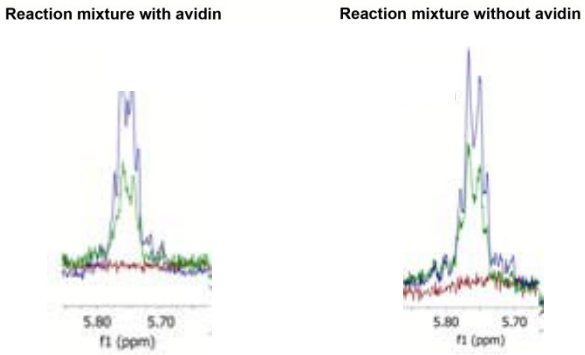


Figure 2: A details of the ^{1}H NMR spectra of the mixture of modified biotin **5a/5b** and cycloocta-1,2,3-selenadiazole **4** before reaction (red line), after 1h irradiation (green line) and subsequent 1h irradiation (blue line).

Further evidence for the reaction on avidin-bound azide **5b**, was brought by MALDI analyses of the modified protein, where the corresponding appearance of higher-mass compounds was observed (See Figure S54). As the binding constant of avidin-biotin is very high at room temperature (K_D = 10^{-15} mol dm^{-3}),[45] we assume that the reaction of azide immobilized by avidin proceeded on a bound substrate and not on a substrate temporarily released from the protein.

Interestingly the conversion of 1,2,3-selenadiazole **4** for both reactions with substrate **5a** (with and without presence of avidine) was possible to enhance by the irradiation for additional one hour. The intensity of the newly formed signals described above significantly increased (about 2-fold), whereas the signals at approximately 3.17, 3.44 and 4.35 ppm nearly disappeared (Figures 2, S51 and S52). We assume that the conversion in this case was higher, because the selenium as internal filter was formed in low amounts because of lower concentration of selenadiazole.

According to these observations, the conjugation reaction between the cyclooctyne analogue obtained from the

corresponding 1,2,3-selenadiazole derivative and the biotinylated azide immobilized by avidin proceeds successfully. In summary, conjugation reactions of cyclooctyne, which was photochemically generated in situ from cycloocta-1,2,3-selenadiazole, with various azides at room temperature, provided satisfactory yields of the corresponding 1,2,3-triazole derivatives as the reaction products. The reaction was successfully tested for the labeling of an avidin-biotin complex, which predestines cyclooctaselenadiazole derivatives for applications in molecular biology. A simple preparation and the chemical stability of 1,2,3-selenadiazoles, non-toxicity of released selenium[44] and room-temperature photochemistry bring a much higher benefit for this method compared with current applications of cyclooctyne systems, the preparations of which are too complex. Although the conversion of 1,2,3-selenadiazole is not quantitative, it can be sufficient for application in various conjugation reactions, such as a labeling of biomolecules.

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