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Readily Accessible Ambiphilic Cyclopentadienes for Bioorthogonal Labeling

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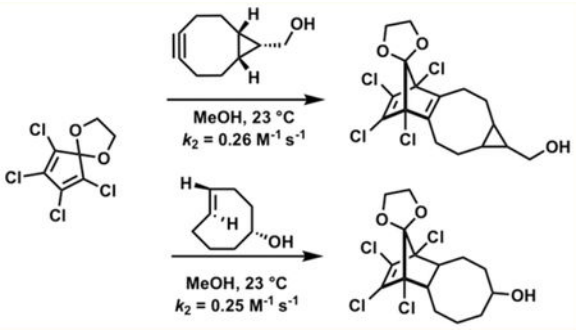
Abstract

A new class of biorthogonal reagents based on the cyclopentadiene scaffold is described. The diene 6,7,8,9-tetrachloro-1,4-dioxospiro[4,4]nona-6,8-diene (a tetrachlorocyclopentadiene ketal, TCK) is ambiphilic and self-orthogonal with remarkable stability. The diene reacts rapidly with a *trans*-cyclooctene and an *endo*-bicyclononyne, but slowly with dibenzo-azacyclooctyne (DIBAC), allowing for tandem labeling studies with mutually orthogonal azides that react rapidly with DIBAC. TCK analogues are synthesized in three steps from inexpensive, commercially available starting materials.

GRAPHICAL ABSTRACT



INTRODUCTION

Bioorthogonal reactions enable the study of biomolecules in living systems for the elucidation of biological processes.[1] The strain-promoted 3+2 azide−alkyne cycloaddition (SPAAC) developed by Bertozzi,[2] and the inverse-electron-demand tetrazine *trans*-

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cyclooctene Diels−Alder reaction introduced by Fox,[3] are bioorthogonal cycloadditions that have been utilized to study complex interactions within biological settings. These reactions take place rapidly and selectively under physiological conditions while avoiding reactions with nucleophiles present in cellular systems.

Research focused on improving the reactivity of the SPAAC and the inverse-electron-demand Diels−Alder (IED-DA) reactions has mostly centered around modifications of the two- π-electron (dienophile or dipolarophile) component. Scheme 1 shows the reactivity of benzyl azide (1) with some of the cyclooctynes developed for bioorthogonal applications. The introduction of a propargylic fluoride (MOFO)[4] on the cyclooctyne scaffold doubles the reactivity of the cyclooctyne, while incorporation of a second electron-deficient fluorine atom at the propargylic position (DIFO)[5] results in a 30-fold increase in reactivity. The accelerated reactivity arises from negative hyperconjugation of the σ^*_{C-F} stabilizing the transition state.[6,7]

Theoretical work by the Alabugin group guided the design of SNO-OCTs (sulfur-, nitrogen-, and oxygen-containing heterocyclic cyclooctynes), where the propargylic heteroatom is endocyclic and antiperiplanar to the alkyne π-bond to maximize the stabilizing effect of the $\pi-\sigma^*$ hyperconjugative interaction.[8,9] Tomooka and co-workers synthesized cycloalkynes with endocyclic heteroatoms and confirmed that the rate enhancement associated with an endocyclic propargyl heteroatom exceeds that of exocyclic propargyl substitution.[8] The hyperconjugative interaction involving the endocyclic heteroatom is stronger because it is antiperiplanar to the reactive π-bond, while the heteroatoms in MOFO and DIFO are gauche.[8] While modulating the electronic properties of the cycloalkyne has improved reactivity, the most reactive cyclooctynes are highly strained multicyclic cyclooctynes such as dibenzocyclooctyne (DIBO),[10] biarylazacyclooctynone (BARAC),[11] dibenzoazacyclooctyne (DIBAC),[12] and *endo*-9-hydroxymethylbicyclo- [6.1.0]nonyne (BCN).[13] Optimization of the azide cyclooctyne cycloaddition has led to rate constants that have leveled off near 1 M^{-1} s^{-1}. For more rapid rate constants, the tetrazine ligation can be used.[3]

Tetrazines are electron-deficient, highly reactive dienes that undergo IED-DA reactions with strained dienophiles to label biomolecules of interest.[14,15] The rate constants for IED-DA reactions of 3,6-di-2-pyridyl-1,2,4,5-tetrazine (2) with a series of dienophiles are shown in Scheme 2. In the IED-DA reaction, the low-lying LUMO of the highly electrophilic tetrazine interacts with the HOMO of the nucleophilic and strained dienophile. Dienophiles with higher lying HOMOs are more reactive toward tetrazines. For example, *trans*-cyclooctene has a higher HOMO energy than cyclooctyne and is more reactive toward tetrazines.[16] An interesting exception is in the series of cycloalkenes from cyclopropene to cyclohexene: the reactivity diminishes despite the increasing HOMO energies across the series.[14,15,17] Recent analyses by our group and the Bickelhaupt group showed that differences in the strength of the secondary orbital interactions, which are especially strong with cyclopropene and weaken with increasing cycloalkene ring size, overcome the differences in primary orbital interactions.[18,19]

The tetrazine−trans-cyclooctene reactions are among the fastest bioorthogonal cycloadditions, with rates exceeding 10^4 M^{-1} s^{-1}.[3] Cyclooctynes (CO),[16] norbornenes (NB),[20] and cyclopropenes (1,3-CP)[21,22] have been paired with tetrazines when a more stable dienophile is required, but these reactions are considerably slower (Scheme 2). Bulky dienophiles, such as 3,3-disubstituted cyclopropenes (3,3-CP)[23] and 3,3,6,6-tetramethylthiacycloheptynes (TMTH),[24] react poorly with tetrazines. These bulky dienophiles react with azides and allow for tandem labeling studies with tetrazine−*trans*-cyclooctene reactions for multitarget imaging.[25] The use of tetrazines in bioorthogonal chemistry is hampered by their bulkiness and vulnerability to nucleophilic attack from biological nucleophiles.[26] To address these issues, the Prescher group has developed bioorthogonal reactions with the less reactive 1,2,4-triazine scaffold.[27]

New reactions are continually being developed that enable rapid, selective ligations to study molecules in chemically complex environments.[28,29] Cyclopentadiene is a classic diene that was used by Diels and Alder in their seminal 1928 publication on the reaction that is now known by their names.[30] Substituted cyclopentadienes have since enjoyed much success in synthesis,[31,32] material functionalization,[33,34] bioconjugation,[35] and chemical trapping,[36,37] yet their potential in bioorthogonal chemistry remains unexplored. We have used computational screening to probe the reactivity of cyclopentadienes with bioorthogonal 2π cycloaddends to design a cyclopentadiene-based bioorthogonal reaction. This method of screening reduces the toil of tedious large-scale reactivity screenings in the laboratory and vastly accelerates the discovery of new bioorthogonal reactions by providing a short list of promising cyclopentadiene-based bioorthogonal reactions to study experimentally.

Cyclopentadiene reacts as both a diene and a dienophile in the Diels−Alder reaction and readily dimerizes at room temperature.[38] Highly substituted cyclopentadienes, such as hexachlorocyclopentadiene, are reactive as dienes but do not readily dimerize at room temperature.[39,40] Dienes with this lack of self-reactivity are referred to as “self-orthogonal”. Substituents at the 1,2 and 3,4 positions of cyclopentadiene sterically impede dimerization by clashing with the substituents at the 5-position of the cyclopentadiene. Extensive experimental and computational studies by the Schleyer group on the stability of 5-substituted cyclopentadienes demonstrate that electronegative substituents destabilize the cyclopentadiene by inducing 4π antiaromatic electron delocalization, whereas electropositive substituents stabilize the cyclopentadiene by creating 6π aromatic charac-ter.[41–43] Our group expanded upon Schleyer’s work with a computational study that predicts that the Diels−Alder reactivity of the cyclopentadiene is tunable through substitution at the 5-position, and that electronegative substituents accelerate the reactivity.[44,45]

Sauer’s pioneering studies on the synthesis and reactivities of 1,2,4,5-tetrazines with many dienophiles have provided inspiration for the design of a variety of reactions in bioorthogonal chemistry.[14,15] Sauer’s detailed reports on the synthesis and reactivities of substituted cyclopentadienes, however, have gone relatively unrecognized.[39,40] The highly reactive, self-orthogonal, and ambiphilic properties of the tetrachlorocyclopentadiene ketal (TCK) shown in Scheme 3 and described by Sauer attracted our attention to it as a potential bioorthogonal diene.[39,40] Ambiphilic molecules can react as electrophiles or nucleophiles, depending on the reaction partner. TCK, being ambiphilic, is able to react with both

electron-deficient dienophiles, such as maleic anhydride, and electronrich dienophiles, such as (1Z,5Z)-cycloocta-1,5-diene.[46] The highly substituted nature of TCK impedes dimerization. TCK is stable at room temperature and requires heating to 80 °C in toluene for 11 days to form a 71% yield of the TCK Diels−Alder dimer.[37]

RESULTS AND DISCUSSION

We have probed computationally the bioorthogonal potential of the TCK with bioorthogonal 2π scaffolds. The M06–2X[47] functional with the 6–31G(d) basis set was used for geometry optimizations. Single-point energies were calculated with the 6–311++G(d,p) basis set, and solvation effects of water were included through use of the conductor-like polarizable continuum model (CPCM).[48,49] Figure 1 shows the computed transition-state structures and activation free energies for the Diels−Alder reactions of TCK with the bioorthogonal cycloaddends of *trans*-cyclooctene (**TS-TCO**), *endo*-bicyclononyne (**TS-BCN**), cyclooctyne (**TS-CO**), norbornene (**TS-NB**), 3,3-dimethylcyclopropene (**TS-3,3-CP**), and dibenzocyclooctyne (**TS-DIBO**). For the reactions of TCK with *trans*-cyclooctene, dibenzocyclooctyne, and cyclooctyne, the computational screening reveals activation free energies of 18.1−20.6 kcal/mol, indicating their potential as viable partners with TCK in bioorthogonal cycloadditions. By contrast, the activation free energies for the Diels−Alder reactions of TCK with norbornene, 3,3-dimethylcyclopropene, and dibenzocyclooctyne range from 23.1 to 27.7 kcal/mol and are too high for bioorthogonal applications. These latter scaffolds are highly reactive with some 1,3-dipoles, providing an opportunity to develop mutually orthogonal cycloadditions.

EXPERIMENTAL SECTION

To test our *in silico* predictions and evaluate the potential of TCK as a bioorthogonal reaction partner, the second-order rate constants of TCK with BCN and TCO cycloaddends were measured experimentally. We chose BCN and TCO as the cycloaddends because they were predicted to be the most reactive bioorthogonal dienophiles toward TCK from the computational screening (Figure 1). TCK was prepared from commercially available hexachlorocyclopentadiene according to Chang's protocol shown in Scheme 4.[46] Hexachlorocyclopentadiene 3 was treated with potassium hydroxide and ethylene glycol to yield TCK. TCK undergoes a rapid 4+2 cycloaddition with BCN and the axial 5-hydroxy-*trans*-cyclooctene (TCO-OH) stereoisomer to give cyclo-adducts 4 and 5, respectively. The reactions of TCK with BCN and TCO-OH give a mixture of four and two stereoisomers, respectively.

Rate constants were measured with ultraviolet−visible (UV/vis) spectroscopy by monitoring the disappearance of the TCK absorption peak under pseudo-first-order conditions. The experimentally observed second-order rate constants for the Diels−Alder reactions of TCK with BCN and TCO-OH in methanol are 0.26 and 0.25 M^{-1} s^{-1}, respectively (Figure 2). These rate constants are comparable to those obtained in previously reported SPAAC bioorthogonal labeling approaches shown in Scheme 1.

Mutually orthogonal bioorthogonal reactions allow for dual labeling studies that monitor multicomponent biological processes by targeting multiple biomolecules.[22] Computational screening predicts that DIBO derivatives will react poorly with TCK. To test this prediction, TCK and DIBAC were stirred together for 20 h at room temperature, and no cycloaddition products were observed. 1,3-Dipoles such as azides react quickly with DIBAC, and poorly with TCO derivatives as shown in Scheme 5.[12,50] Scheme 5 outlines these findings and demonstrates how tandem labeling is possible with the mutually orthogonal TCK−TCO and benzyl azide−DIBAC reactions.

Scheme 6 illustrates a three-step protocol for the synthesis of an *N*-hydroxysuccinimide-functionalized TCK to enable bioconjugation to primary amines. Ketalization of hexachlorocyclopentadiene 3 was carried out with (±)-1,2,4-butanetriol to yield the intermediate alcohol 6. Oxidation to the corresponding carboxylic acid 7 and subsequent coupling to *N*-hydroxysuccinimide under standard conditions afforded the desired activated ester 8.

To validate the biocompatibility of the reaction, the commercial neuropeptide [D-Ala^2,D-Leu^5]-enkephalin, used to prevent neuronal damage against hypoxic or ischemic induced brain injury, was chosen for initial labeling experiments.[51] Activated diene 8 was readily conjugated to [D-Ala^2,D-Leu^5]-enkephalin via the succinimidyl ester to afford cyclopentadiene−peptide conjugate 9. Peptide conjugate 9 efficiently underwent the Diels−Alder cycloaddition with Cy5-TCO at ambient temperature to afford the fluorescent peptide 10, as shown in Scheme 7.

Many bioorthogonal reagents are sensitive to air or light, react with biological endogenous nucleophiles, such as thiols, or are unstable as a result of strain, making prolonged labeling studies and storage difficult.[11,28,52] TCK can be stored at room temperature as a white solid with a melting point range of 64.5−65.5 °C. No decomposition or dimerization of TCK was observed by proton NMR after stirring for 63 hours at 37 °C in a 1:1 CD_3CN:D_2O mixture with cysteine. TCK is stable in aqueous media and inert to the nucleophilic thiol cysteine. Although TCK is insoluble in water, polar groups that increase solubility can be added.

CONCLUSION

With the aid of *in silico* computational screening, we report TCKs as a new class of bioorthogonal reagents with reaction rates toward *endo*-BCN and TCO-OH that are practical for biological labeling studies. Proof of fluorescence peptide labeling with TCK is demonstrated using a commercial neuropeptide and the near-infrared cyanine dye, Cy5. The enduring stability of TCK is ideal for long-term applications and our computational studies suggest future tandem labeling with azide reactions is plausible. TCK is readily synthesized from inexpensive starting materials and stable at room temperature. The dynamic reactivity, accessibility, and stability found in TCKs are essential for its adoption as a bioorthogonal reagent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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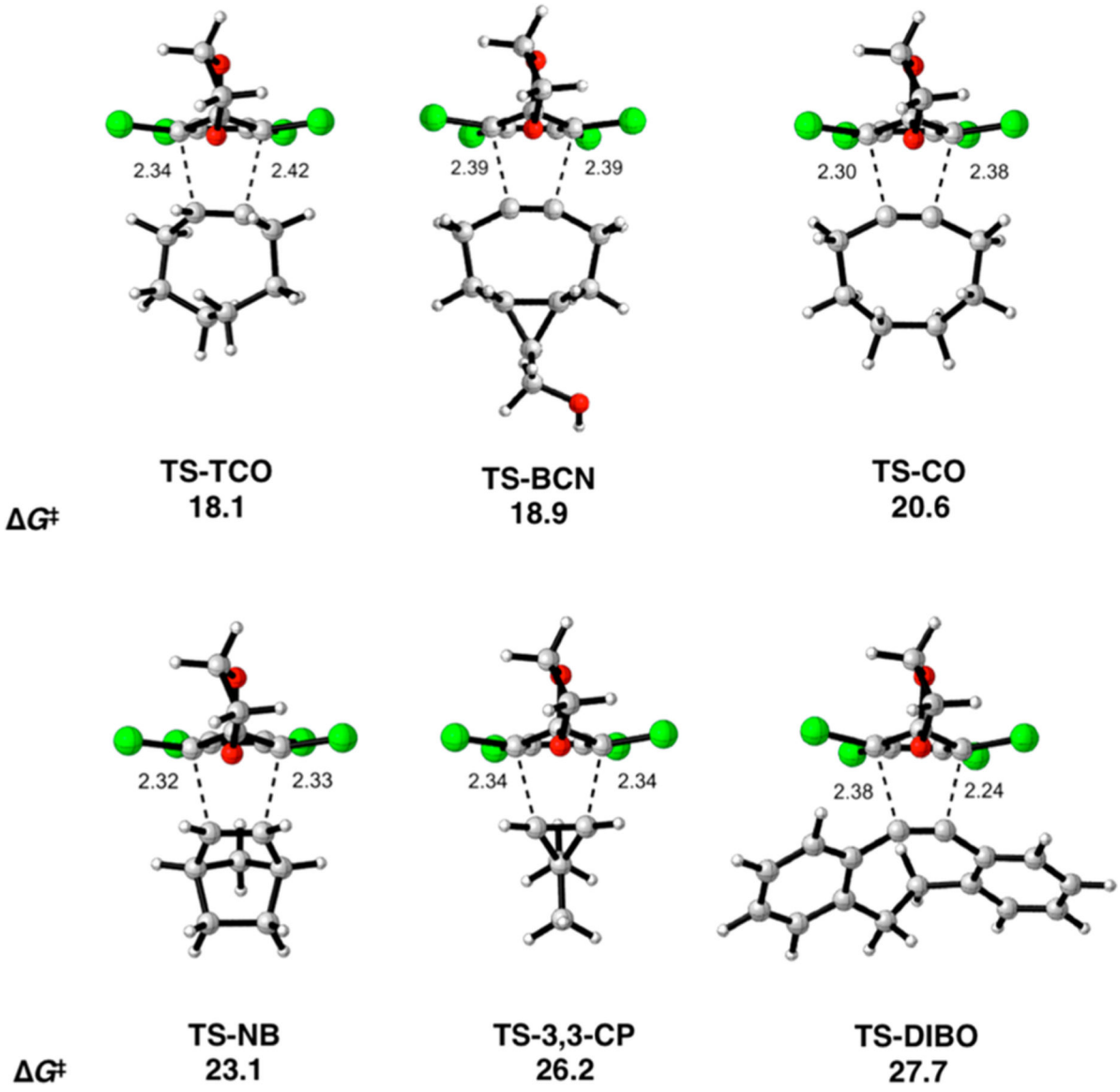


Figure 1.

Transition-state structures and activation free energies (in kcal/mol) for the Diels−Alder reactions of TCK with bioorthogonal cycloaddends.

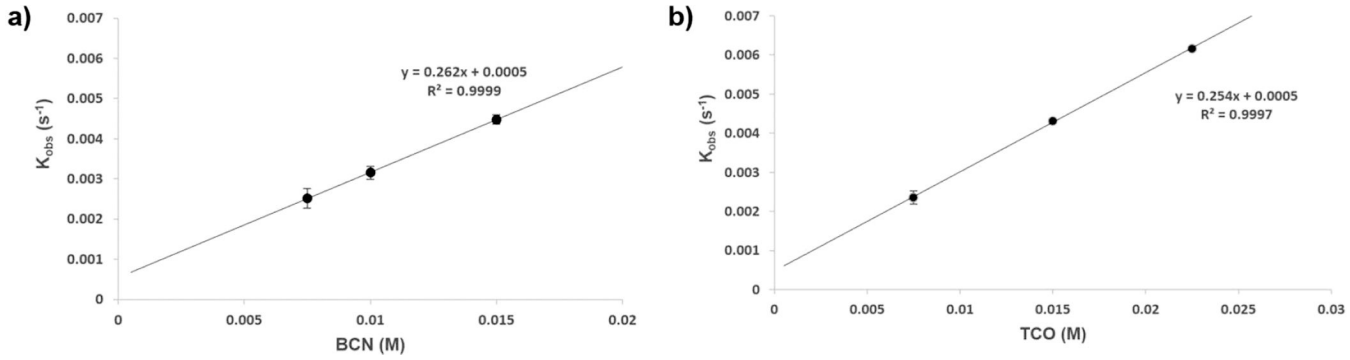
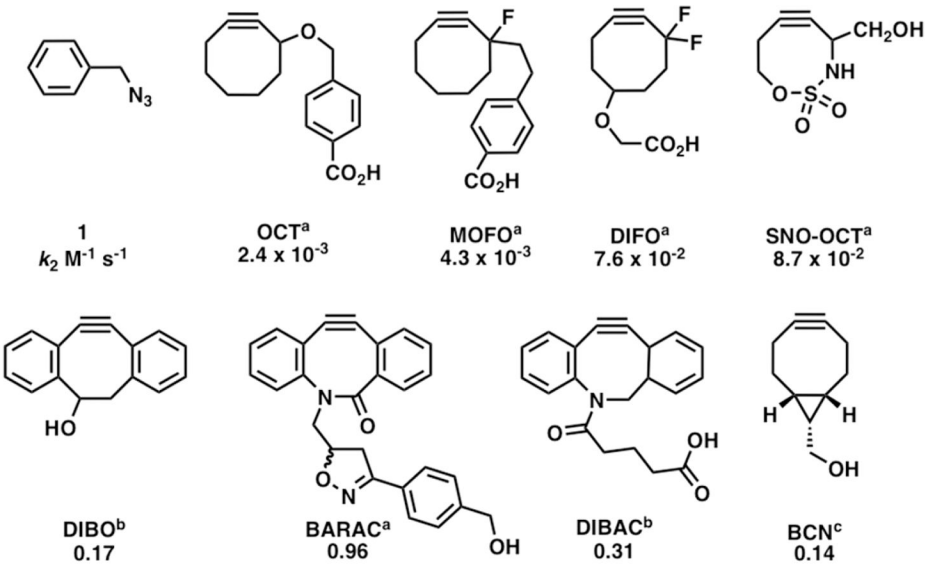


Figure 2.

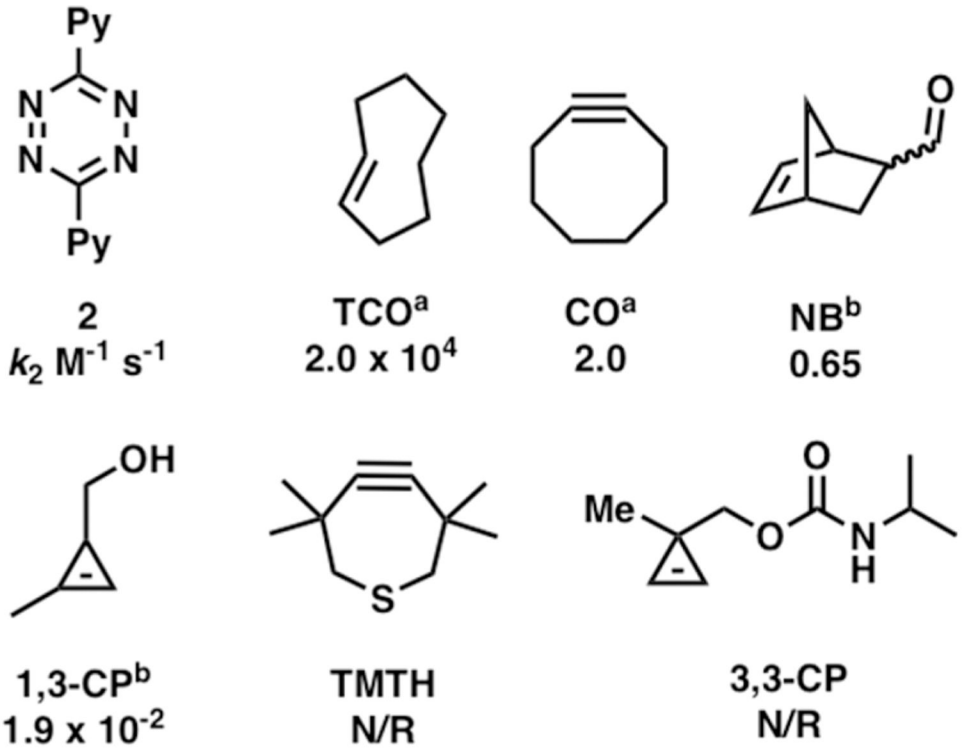
(a) Plot of rate observed vs concentration of BCN, with the slope taken as the second-order rate constant. (b) Plot of rate observed vs concentration of TCO-OH, with the slope taken as the second-order rate constant.



[a]Reaction rates were measured in [a]acetonitrile (CD_3CN), [b]methanol (CD_3OD), or [c]3:1 CD_3CN/D_2O at ambient temperature.

Scheme 1.

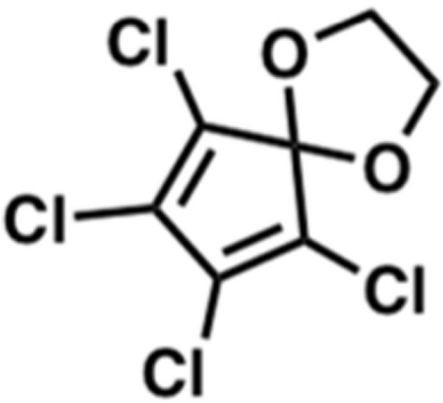
Cyclooctynes and Second-Order Rate Constants (M^{-1} s^{-1}) for Reactions with Benzyl Azide (1)[a]



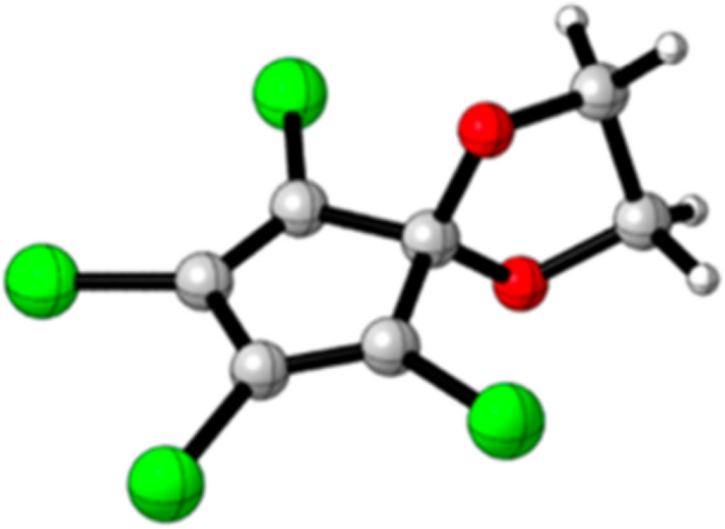
[a]Reaction rates were measured in [a]9:1 MeOH/H_2O or [b]MeOH at ambient temperature. N/R indicates no reaction.

Scheme 2.

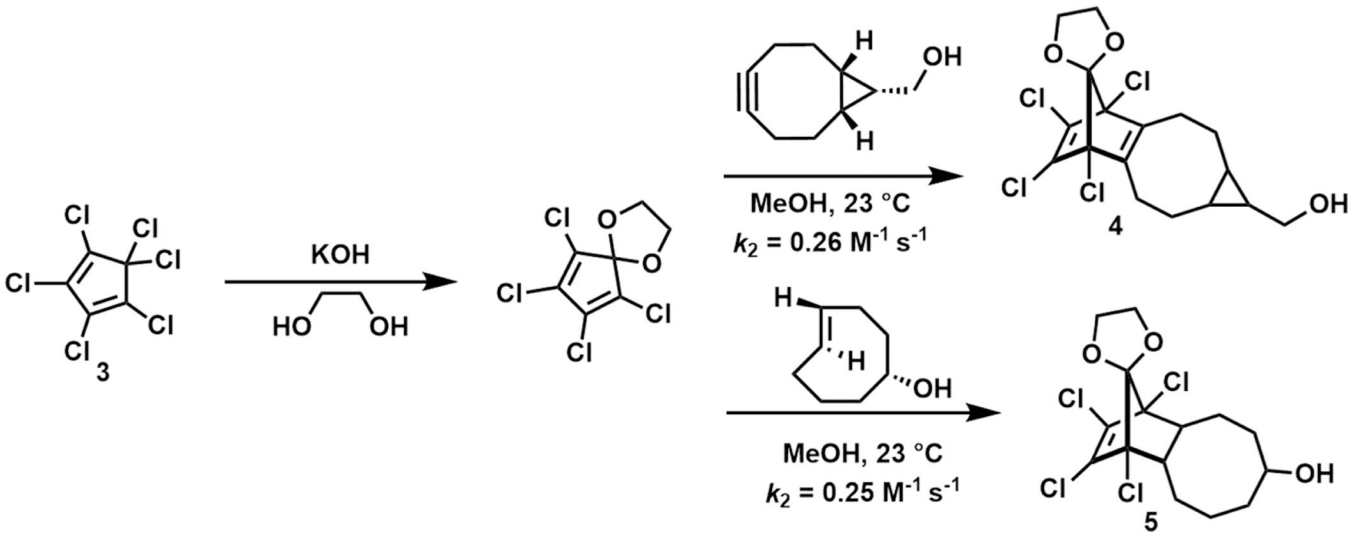
Dienophiles and Their Second-Order Rate Constants for Reactions with 3,6-Di-2-pyridyl-1,2,4,5-tetrazine (2)[a]



TCK

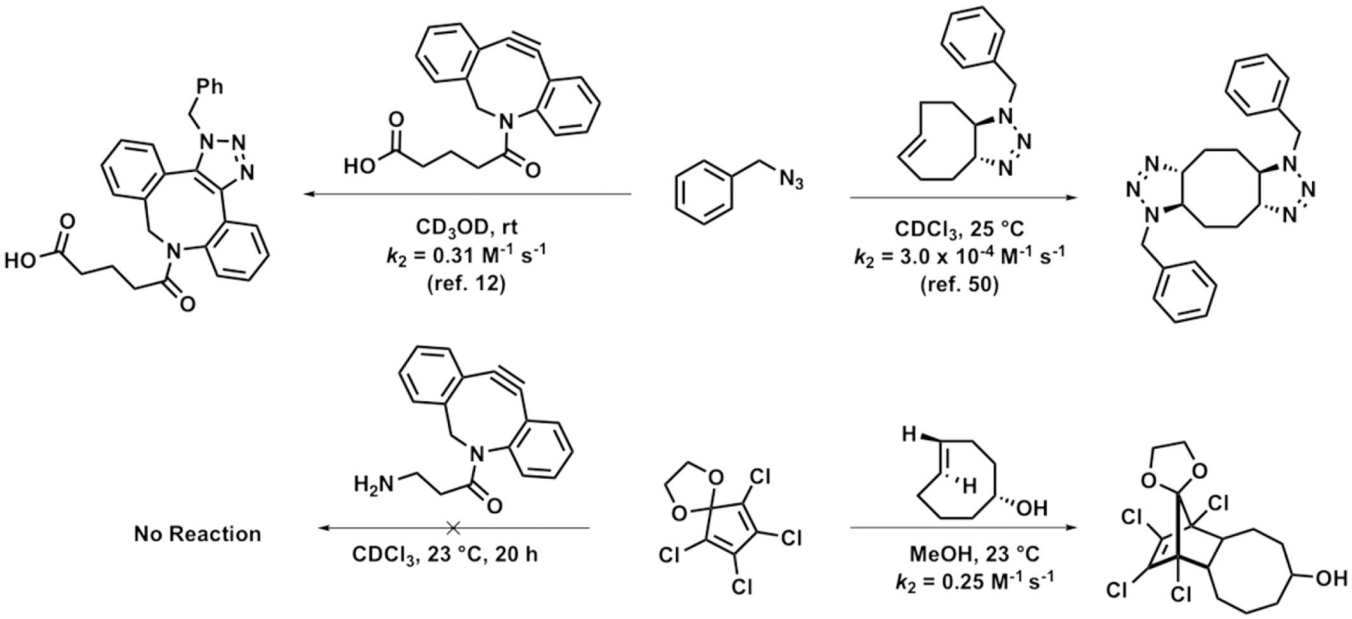


Scheme 3.
Tetrachlorocyclopentadiene Ketal (TCK)



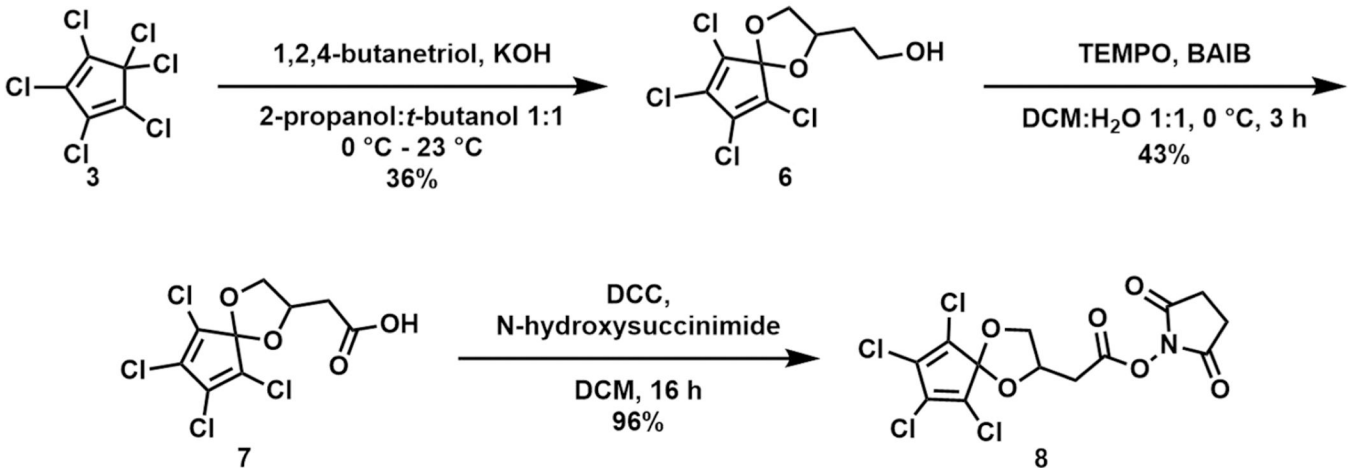
Scheme 4.

Synthesis of TCK and Cycloaddition Rates with TCO-OH and BCN



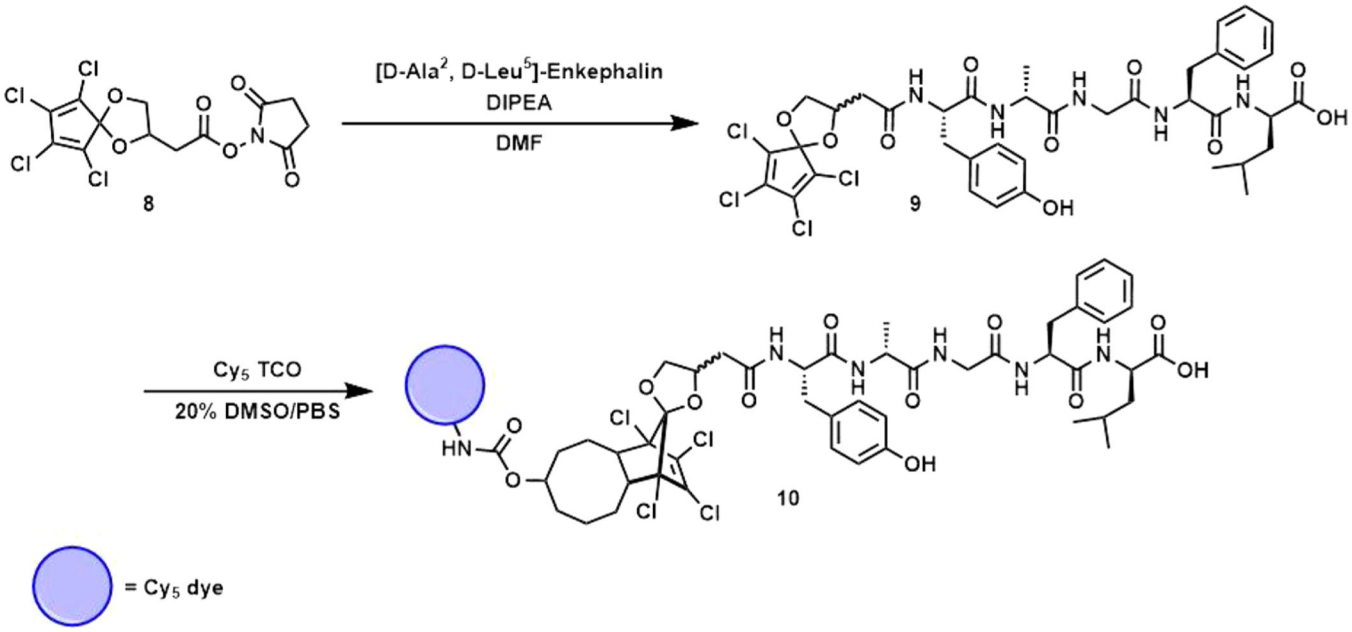
Scheme 5.

Mutual Orthogonality between the TCK TCO-OH and Benzyl Azide−DIBAC Reactions



Scheme 6.

Synthesis of TCK Succinimidyl Ester 9 for Bioconjugation to Primary Amines



Scheme 7.
Bioconjugation and Fluorescence Labeling of [D-Ala^2,D-Leu^5]-Enkephalin