

A Journal of the Gesellschaft Deutscher Chemiker

Angewandte Chemie

GDCh

International Edition

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Accepted Article

Title: General, Divergent Platform for Diastereoselective Synthesis of trans-Cyclooctenes with High Reactivity and Favorable Physiochemical Properties

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This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). This work is currently citable by using the Digital Object Identifier (DOI) given below. The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.202101483

Link to VoR: <https://doi.org/10.1002/anie.202101483>

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General, Divergent Platform for Diastereoselective Synthesis of *trans*-Cyclooctenes with High Reactivity and Favorable Physicochemical Properties

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Abstract: *trans*-Cyclooctenes (TCOs) are essential partners for the fastest known bioorthogonal reactions, but current synthetic methods are limited by poor diastereoselectivity. Especially hard to access are hydrophilic TCOs with favorable physicochemical properties for live cell or *in vivo* experiments. Described is a new class of TCOs, 'a-TCOs', that is prepared in high yield via stereocontrolled 1,2-additions of nucleophiles to *trans*-cyclooct-4-enone, which itself was prepared on large scale in two steps from 1,5-cyclooctadiene. Computational transition state models rationalize the diastereoselectivity of 1,2-additions to deliver a-TCO products, which were also shown to be more reactive than standard TCOs and less hydrophobic than even a *trans*-oxocene analog. Illustrating the favorable physicochemical properties of a-TCOs, a fluorescent TAMRA derivative in live HeLa cells was shown to be cell-permeable through intracellular Diels-Alder chemistry and to washout more rapidly than other TCOs.

Introduction

Bioorthogonal reactions are a class of rapid, selective reactions that can proceed efficiently and selectively in biological systems without interfering with biological functional groups.^[1] Bioorthogonal chemistry has enabled a deeper understanding of native biological processes and expanded the frontiers of chemical biology through innovations in nuclear medicine,^[2] drug delivery,^[3] and biomaterials.^[4] The tetrazine ligation with *trans*-cyclooctenes (TCOs) has been at the forefront of bioorthogonal methodologies due to rapid reaction kinetics that generally exceed k_2 10^4 M⁻¹s⁻¹.^[5]

TCO derivatives are synthesized by a photochemical flow-method under singlet sensitized conditions, driving an otherwise unfavorable isomeric ratio in favor of the *trans*-isomer via selective metal complexation to Ag(I).^[6] Flow photoisomerization has been carried out on scales as large as 20 grams,^[7] and protocols have been developed where flow is mimicked by periodically stopping irradiation and capturing TCO product by filtering through AgNO₃-silica and resubjecting the filtrate to photoisomerization (254 nm).^[8] While this method has been used to synthesize a large number of different TCO derivatives,^[9] the production of diastereomers due to the planar chirality of the alkene presents a bottleneck for the majority of TCO syntheses.

For example, the most frequently utilized TCO derivatives are the axial and equatorial diastereomers of 5-hydroxy-*trans*-cyclooctene, which are produced through photoisomerization of 5-hydroxy-*cis*-cyclooctene in 72%.^[6] Derivatives of the axial diastereomer have emerged as especially useful, as they are an order of magnitude more reactive than equatorial diastereomers,^[5,10] can promote fluorogenic effects for cell imaging,^[11] and have been employed in 'click-to-release' strategies for bioorthogonal uncaging.^[3a,3b] However, photoisomerization produces the equatorial:axial isomers in 2.2:1 ratio, and thus produces $\leq 24\%$ of the axial diastereomer. A modified procedure using Ag(I) sulfonated silica gel slightly improves the yield of the axial diastereomer to $\leq 27\%$.^[12] Even for the equatorial diastereomer, the need to separate diastereomers represents a limitation for material throughput. For other TCO derivatives, chromatographic separation of diastereomers can be very difficult and not feasible by flash chromatography.^[13] One solution to the diastereomer issue has been to utilize s-TCO—a highly strained dienophile prepared from the meso compound precursor.^[14] s-TCO is most reactive dienophile known, and is suitable for applications as a probe molecule in bioorthogonal chemistry,^[3e,15] but due to alkene isomerization, s-TCO is often unsuitable as a reporter where more prolonged cellular incubation is required.^[16] Currently, several TCO derivatives are available for purchase, but they are very expensive. A general and diastereoselective synthesis of TCO derivatives could greatly increase the availability of these useful compounds for chemical biology research.

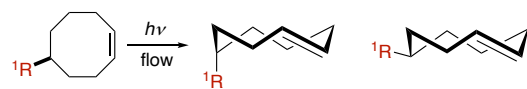
In addition to stereoselectivity, hydrophobicity has been a longstanding issue with TCOs where non-specific binding and extensive wash-out protocols in live cell assays are undesirable consequences.^[17] Recently, heterocyclic *trans*-cyclooctenes with backbone oxygen atoms have been shown to have higher hydrophilicity and improved physicochemical properties for cellular and *in vivo* imaging applications.^[13,18] However, a drawback for these oxo-TCOs are lengthy syntheses that produce diastereomers that can be separated only with difficulty.

Described here is a diastereoselective method for synthesizing TCOs with favorable physicochemical properties in high yield and useful scale through the stereocontrolled additions of nucleophiles to *trans*-cyclooct-4-enone **2** (Figure 1). As **2** lacks a stereocenter, it therefore can be prepared on large scale using

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Previously

- Poor diastereoselectivity
- Separation bottleneck



This work

- Single Diastereomer
- Divergent synthesis
- Improved hydrophilicity
- Facile purification

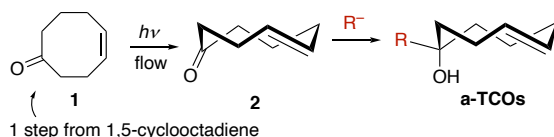


Figure 1. Previous approach to *trans*-cyclooctene syntheses compared to the general, divergent, and diastereoselective platform described in this paper.

flow photochemistry from *cis*-cyclooct-4-en-1-one (**1**) without the complication of diastereoselectivity in the photoisomerization step encountered previously. The rigid structure of **2** distinguishes the faces of the ketone, and computation was used to predict that nucleophilic additions to **2** take place exclusively from the equatorial-face of the ketone to produce axial products as single diastereomers. By engaging a range of nucleophiles, ketone **2** serves as a general platform for preparing a range of functionalized axial-5-hydroxy-*trans*-cyclooctene ('a-TCO') analogs. The method provides improved access to new compounds as well as known TCO derivatives required for *in vivo* radiochemistry,^[10] click-to-release chemistry,^[2a,3a,3e] and sulfenic acid detection in live cells.^[19] An a-TCO derivative was shown to be more reactive than both *axial* and *equatorial* diastereomers of 5-hydroxy-*trans*-cyclooctene as well as oxo-TCO in cycloadditions with tetrazines. As a demonstration of the favorable physicochemical properties of a-TCOs, a fluorescent TAMRA derivative was shown to be cell-permeable by demonstrating intracellular Diels-Alder reactions in live cells and was shown to washout of HeLa cells more rapidly than even a hydrophilic oxo-TCO analog.

Results and Discussion

Ketone **1** was prepared simply in a single step by the Wacker oxidation (5 mol% Pd(OAc)₂, AcOH, benzoquinone) of 1,5-cyclooctadiene **3** on multigram scale (Figure 2A).^[20] Alternately, **1** can be prepared in a single step by Dess-Martin oxidation of 5-hydroxy-*cis*-cyclooctene.¹⁹ Photochemical isomerization of **1** was conducted using the general photochemical flow method.^[6,9] Using a small cartridge and bed of capture silica, ketone **2** was prepared in 62% yield and at a rate of ~150 mg/h, and in a typical workflow, ketone **2** was prepared in 2.5 g batches. Computation was used to predict if nucleophilic addition to TCO **2** would be diastereoselective (Figure 2B). It was reasoned that 1,3-diaxial interactions in the lowest energy 'crown' conformation of the parent TCO would favor addition to the equatorial face by nucleophiles, akin to the facial selectivity of conformationally biased cyclohexanones.^[21] The barriers for the reaction of LiAlH₄ with **2** were calculated at the M06L/6-311+G(d,p) level using a SCRf solvent model for THF (Figure 2C). The calculated barriers relative to the pre-reaction complex for the equatorial attack by hydride are ΔG^\ddagger 14.09 kcal mol⁻¹ and ΔH^\ddagger 12.00 kcal mol⁻¹. The

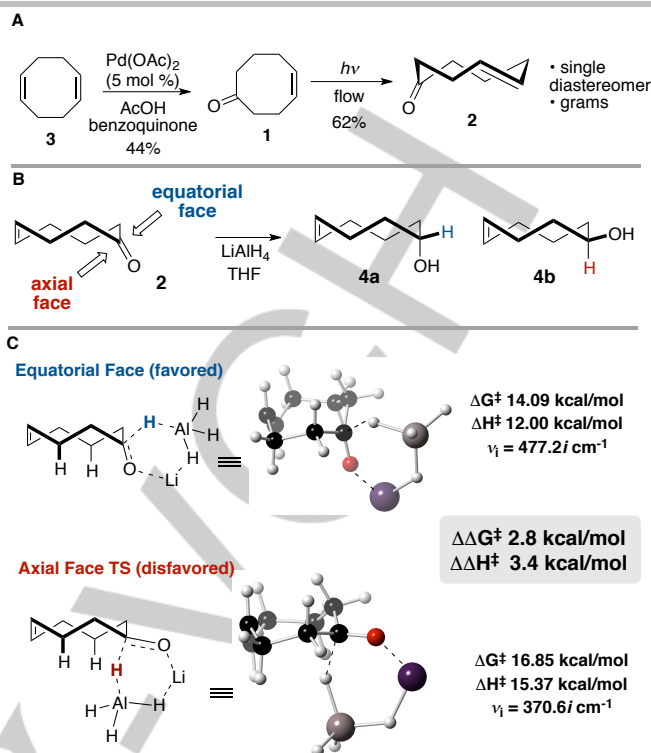
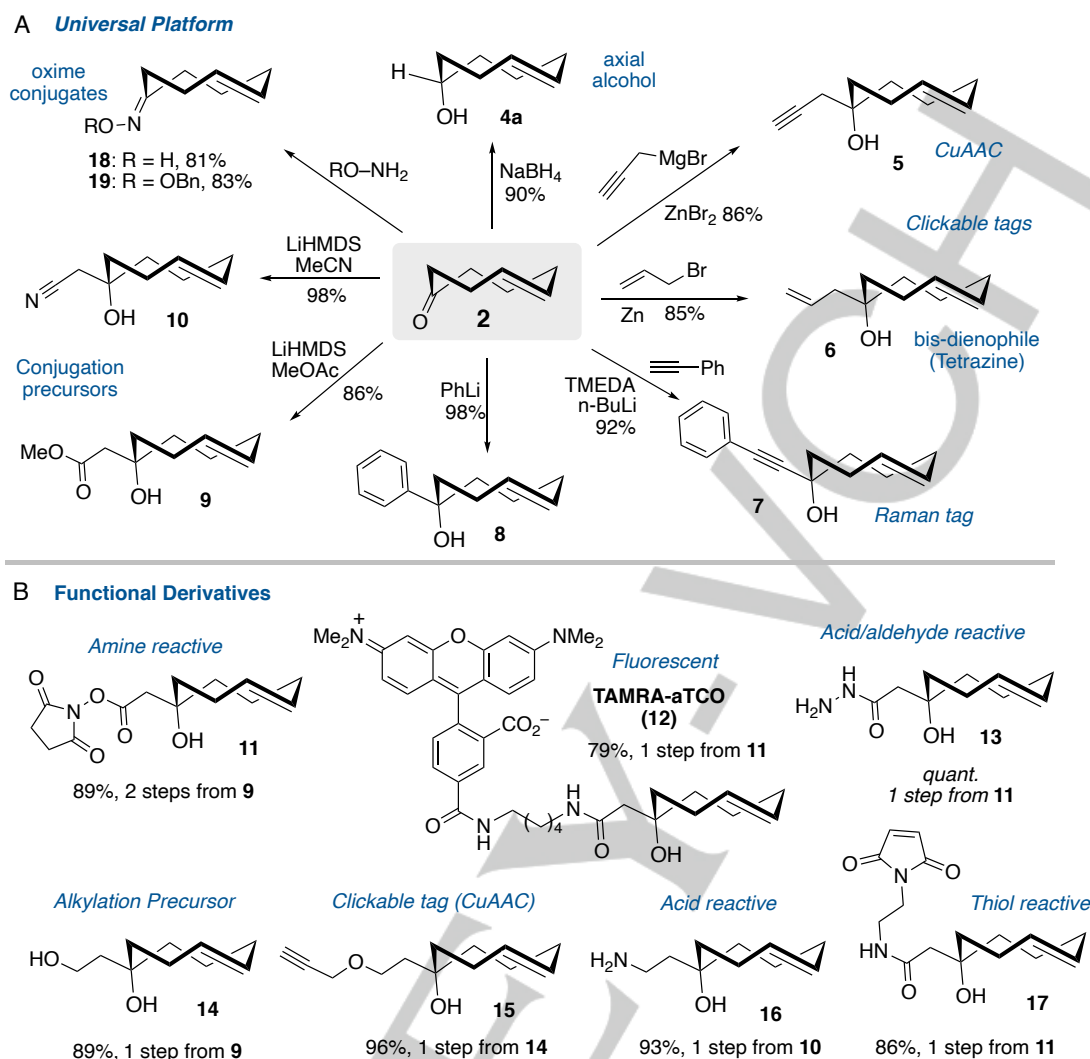


Figure 2. (A) Synthesis of *cis*-cyclooct-4-en-1-one **1** and *trans*-cyclooct-4-en-1-one **2**. (B) Reaction of *trans*-cyclooct-4-en-1-one **2** with LiAlH₄. Nucleophilic addition can occur to the equatorial or axial face of **2** producing diastereomers **4a** and **4b**, respectively. (C) Transition state calculations predict nucleophilic addition to equatorial face would be favored over the axial face.

barrier is significantly lower than that calculated for axial attack ($\Delta\Delta G^\ddagger$ 2.8 kcal/mol and $\Delta\Delta H^\ddagger$ 3.4 kcal/mol). Encouraged by these computational predictions, we experimentally investigated additions of nucleophiles to TCO **2** (Scheme 1A). In agreement with the computational data, nucleophilic addition of hydride occurred exclusively to the equatorial face of **2** to produce **4a**. While LiAlH₄ gave **4a** in only 67% yield due partial alkene isomerization, NaBH₄ provided **4a** as a single diastereomer in 90% yield with no isomerization. Derivatives of axial alcohol **4a** are especially useful for their rapid kinetics^[10] and their utility in click-to-release chemistry,^[3e] but previous syntheses of **4a** were low yielding ($\leq 27\%$) and required separation from major diastereomer **4b**.^[6,12] The improved route described here is short, selective and more scalable.

Nucleophilic additions with a diverse array of nucleophiles were preformed to create a library of a-TCOs (Scheme 1). Compound **5** bearing a bioorthogonal alkyne tag was recently developed for the capture of cellular protein sulfenic acids via transannular thioetherification with subsequent proteomic analysis enabled by a CuAAC-chemistry workflow.^[19] We note that **5** (and other a-TCOs) are still suitable for selective bioorthogonal chemistry as they only modify sulfenic acids at relatively high TCO concentration (generally $\geq 500\text{ }\mu\text{M}$), but not under the conditions typically used in intracellular bioorthogonal chemistry (generally $\leq 10\text{ }\mu\text{M}$). In the previous study, **5** was synthesized by direct photoisomerization in only 6% yield and required separation from two isomers. As shown in Scheme 1A, compound **5** can be

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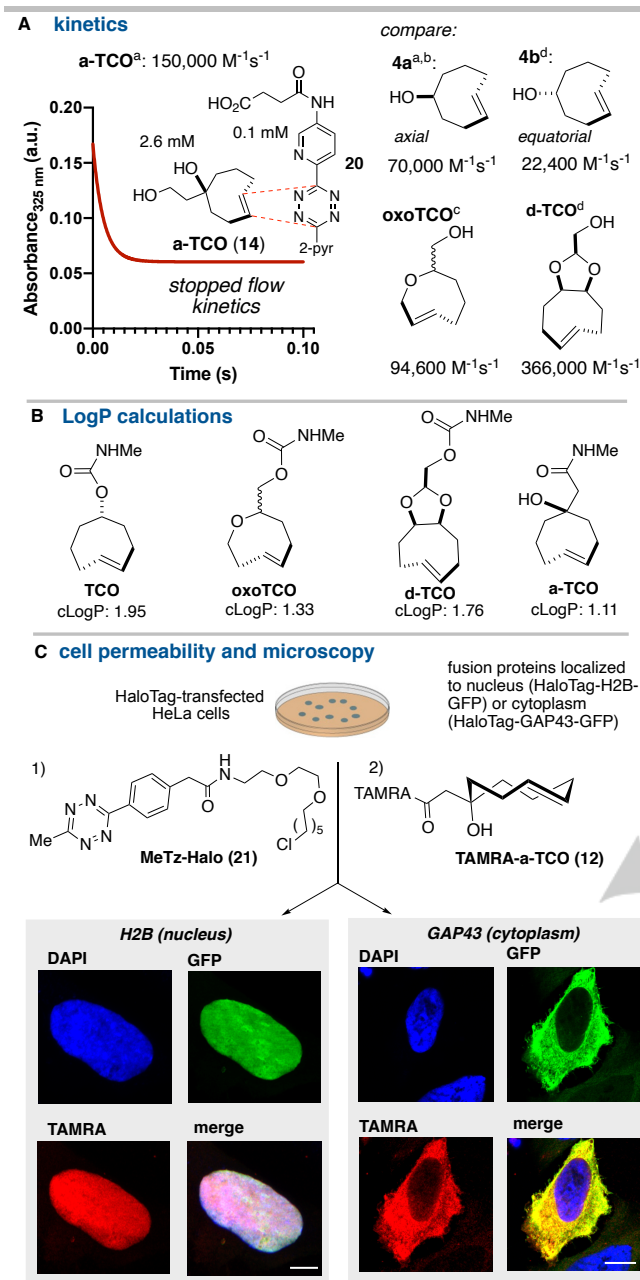
Scheme 1. (A) Nucleophilic addition reactions of *trans*-cyclooct-4-en-2-one (**2**) serves as a universal platform for the diastereoselective synthesis of a-TCOs as well as oxime conjugates. (B) Functional derivatives readily available from conjugation precursors **9** and **10**

prepared in 86% yield as a single diastereomer by adding propargyl magnesium bromide to ketone **2**. Similarly, compound **6** bearing a simple alkene tag can be constructed in 85% yield and as a single diastereomer by the addition of allyl zinc bromide to ketone **2**. Like *trans*-cyclooctenes, simple α -olefins can function as dienophiles in tetrazine ligation but with much slower kinetics, providing handles for potential sequential bioorthogonal chemistry applications.^[22] Organolithium nucleophiles generated *in-situ* were used to synthesize TCOs **7-8**. Illustrating the ability to introduce a tag that may be useful for Raman spectroscopy and imaging,^[23] TCO **7** bearing a phenylacetylene group was synthesized by the addition of lithium phenyl acetylene to **2** in 92% yield. TCO **8** was synthesized similarly by the addition of phenyl lithium to **2** in 98% yield and serves as a model reaction for nucleophilic addition of aryl groups to TCO **2**.

The diastereoselective additions of **2** with methyl α -lithioacetate or lithioacetonitrile provided a straightforward path to introduce handles for conjugation via amide bond or ether formation. The reaction of ketone **2** with LiHMDS/methyl acetate produced ester **9** in 86% yield. Similarly, the combination of **2** with LiHMDS and acetonitrile produced nitrile **10** in 98% yield. Hydrolysis of **9** with

trimethyltin hydroxide^[24] followed by DIC-mediated coupling with N-hydroxysuccinimide gave NHS ester **11** in 89% yield (Scheme 1B). Alternately, using LiOH gave **11** in 46% yield after DIC coupling. Through amide coupling, **11** gave the fluorescent TAMRA-conjugate **12** with favorable physicochemical properties, and **11** was also combined with hydrazine monohydrate to give hydrazide **13** with a potential handle for aldehyde conjugation.^[25] TCO **9** also was combined with LiAlH_4 to provide diol **14** in 89% yield which was next used in a Williamson ether synthesis with NaH/propargyl bromide to give 96% yield of propargyl ether **15**. Compound **15** serves a more stable alternative to alkyne-tagged TCO **6**, which is found to polymerize if not stored in solution. Amine **16** was synthesized by LiAlH_4 reduction of TCO nitrile **10** in 93% yield to introduce an acid reactive conjugation handle. Maleimide **17** was prepared through amide coupling of TCO **11** with *N*-(2-aminoethyl)maleimide in 86% yield. The yield of **17** is significantly higher than what was observed in previous preparations of TCO-maleimides from activated nitrophenylcarbonates.^[14] Furthermore, oxime conjugates **18** and **19** could be prepared by the direct condensation of the corresponding hydroxylamines in the presence of pyridine

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^a k_2 measured in 95:5 water:MeOH. ^bThe reaction of **4a** with a PEGylated amide of **20** in 100% H_2O was previously measured as $k_2 80,200 \text{ M}^{-1}\text{s}^{-1}$.^[5] ^c k_2 previously measured with **20** in 100% H_2O .^[13] ^d k_2 previously measured with PEGylated amide of **20** in 100% H_2O .^[5]

Figure 3. (A) Stopped flow kinetics under pseudo-first order conditions were used to determine second order rate constants for the reactions of tetrazine **20** with **14**, allowing comparison to less reactive **4a** and **4b**. (B) cLogP calculations for a series of analogs of TCO, oxoTCO, d-TCO, and a-TCO illustrate the improved hydrophilicity of a-TCO conjugates. (C) Cell permeability was demonstrated by incorporation of MeTz-Halo (**21**) into HeLa cells transfected with either H2B-HaloTag-GFP (nuclear) or GAP43-HaloTag-GFP (cytoplasmic), followed by labeling with TAMRA-a-TCO (**12**). Confocal microscopy images of transfected cells labeled with TAMRA-a-TCO show subcellular colocalization of GFP and TAMRA fluorescence, consistent with selective intracellular labeling. Scale bars for H2B and GAP43 labeling are 5 μM and 10 μM , respectively.

(Scheme 1A). In summary, ketone **2** can serve as a readily prepared, central intermediate for the diastereoselective preparation of a range of hydrophilic a-TCO conjugates.

a-TCO derivatives also display rapid kinetics in Diels-Alder reactions compared to most other TCOs. The kinetics for the reaction of a-TCO derivative **14** toward a 3,6-dipyridyl-s-tetrazinyl succinamic acid derivative **20** were measured by stopped flow kinetics at 25 °C in 95:5 PBS:MeOH (Figure 3A). With a second-order rate constant of $150,000 \pm 8000 \text{ M}^{-1}\text{s}^{-1}$, a-TCO is more than twice as reactive toward **20** as the axial isomer of 5-hydroxy-*trans*-cyclooctene (**4a**, $70,000 \pm 1800 \text{ M}^{-1}\text{s}^{-1}$), and nearly 7-times more reactive than equatorial isomer **4b** ($22,400 \pm 40 \text{ M}^{-1}\text{s}^{-1}$).^[5] The faster kinetics are likely due an increase in olefinic strain for a-TCO due to steric effect of geminal substitution in the 8-membered ring backbone. Likely for the same reason, similar rate accelerations have been observed for more sterically encumbered derivatives of **4a**.^[10] a-TCO **14** is also more reactive than oxo-TCO,^[13] and the conformationally strained, bicyclic d-TCO is only 2.2-times more reactive than **14**.^[5] As shown in Figure 3B, a-TCO derivatives are also calculated to have improved physicochemical properties relative to other TCO derivatives. While both oxo-TCO and d-TCO were previously introduced as less hydrophobic bioorthogonal reagents, the methylamine conjugate of a-TCO is calculated to have even a lower cLogP value.

The stability of a-TCO **14** is very similar to that of axial-5-hydroxy-*trans*-cyclooctene **4a**, which is used broadly for applications in bioorthogonal chemistry. In MeOD, 35 mM solutions of both **14** and **4a** are >99% stable after 1 week at room temperature. After 24 h at room temperature in D_2O -PBS (pD 7.4), 33 mM solutions of **14** and **4a** display 90% and 85% stability, respectively. In D_2O -PBS containing 25 mM mercaptoethanol, 49% of both **14** and **4a** remained after 20 h.

The fluorescent conjugate TAMRA-a-TCO **12** was shown to be cell permeable through selective bioorthogonal reaction inside live cells using the HaloTag self-labeling platform (Figure 3C). Here, cells are transfected with a GFP-HaloTag construct fused to a protein that controls subcellular localization, and then labeled by a tetrazine-HaloTag ligand **21**. Upon subsequent reaction with fluorescent TAMRA-a-TCO, conjugation is expected only in those cells that express the HaloTag fusion protein, and co-localization of GFP and TAMRA fluorescence is expected. As shown in Figure 3C, HeLa cells were transfected with either HaloTag-H2B-GFP (nucleus) or HaloTag-GAP43-GFP (cytoplasm), labeled with MeTz-Halo (**21**) (10 μM), washed and then treated with TAMRA-a-TCO (1 μM) for 30 min, at which point the TCO reagent was chased by a non-fluorescent tetrazine, and the cells were fixed and imaged. As shown in Figure 3C, for both nuclear and cytoplasmic targets, selective colocalization of the TAMRA signal with GFP was observed in both cases.

The advantage of the increased hydrophilicity for a-TCO conjugates was demonstrated by comparing the washout times for fluorescent derivatives in mammalian cells. While TCO-derivatives offer rapid labeling kinetics, a consideration for labeling by fluorophore-tagged TCOs has been the background fluorescence due to non-covalent cellular binding that can be ameliorated only by extended washout times. More hydrophilic oxo-^[13] and dioxo-*trans*-cyclooctenes^[18a] with backbone oxygens offer improvements, but are difficult to synthesize and, for dioxo-TCO, display reduced Diels-Alder kinetics. As shown in Figure 4, we prepared the fluorescent conjugates TAMRA-TCO, TAMRA-oxo-TCO and TAMRA-a-TCO and compared their cellular washout times to unconjugated TAMRA. Thus, HeLa cells were incubated for 30 min with TAMRA-dyes, and cells were initially

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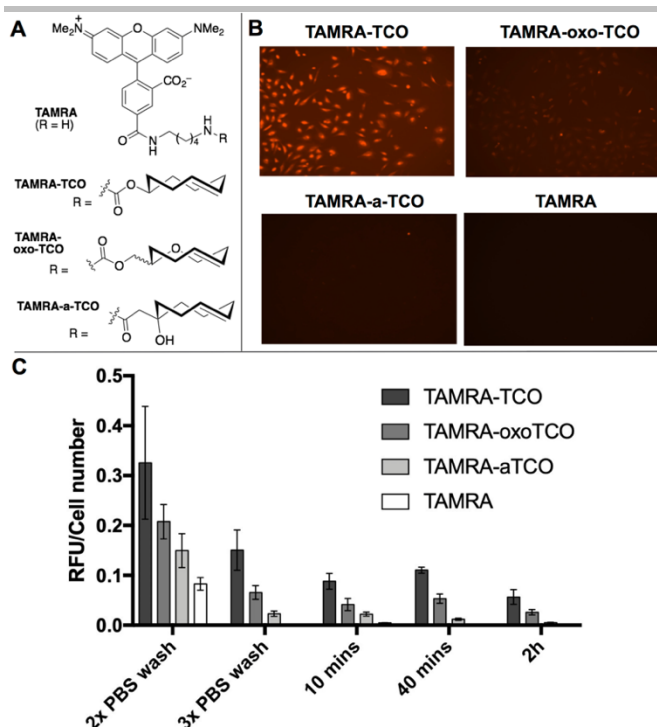


Figure 4. (A) Structures of TAMRA and conjugates with TCO, oxo-TCO and a-TCO. (B,C) HeLa cells were incubated for 30 min with TAMRA-dyes, and cells were initially washed three times with PBS, and then cell media was exchanged after 10, 40 and 120 minutes. After each wash, cells were imaged live by fluorescence microscopy with illumination at 531 nm and with fixed-intensity across all samples. (B) Widefield images cells after 3 washes. (C) Comparison of background fluorescence across all experiments, quantified by dividing total fluorescence by the number of cells in each image.

washed three times with DPBS, and then cell media was exchanged after 10, 40 and 120 minutes. After each wash, cells were imaged live by fluorescence microscopy with illumination at 531 nm and fixed-intensity across all samples. Widefield images of the cells after 3 washes are shown in Figure 4B; images after the earlier and later washings are shown in Figure S10. Background fluorescence was quantified by dividing total fluorescence by the number of cells in each image (Figure 4C). For **TAMRA-TCO**, cells are markedly fluorescent after 3 washings, and still display significant background after washing for 2 hours. The background is improved with **TAMRA-oxo-TCO** and especially with **TAMRA-a-TCO**, which after initial 3x wash shows an 85% reduction in background fluorescence relative to **TAMRA-TCO**. After 2 hours, washout of **TAMRA-a-TCO** is essentially complete with background equivalent to TAMRA itself, whereas **TAMRA-TCO** and **TAMRA-oxo-TCO** both still display residual fluorescence even after 2 hours.

Conclusion

In summary, a-TCOs are a class of *trans*-cyclooctenes with favorable physicochemical properties that can be prepared in high yield through the stereocontrolled additions of nucleophiles to *trans*-cyclooct-4-enone (**2**), a *trans*-cyclooctene that can be prepared on large scale in two steps from 1,5-cyclooctadiene. Computation was used to rationalize diastereoselectivity of 1,2-

additions to deliver a-TCO products. The strategy can be applied to the synthesis of a range of usefully functionalized a-TCOs with high yield, selectivity. a-TCOs were also shown to be more reactive than standard TCOs and less hydrophobic than even hydrophilic oxo-TCO analogs. As a demonstration of the favorable physicochemical properties of a-TCOs, a fluorescent TAMRA derivative was shown to be cell-permeable by demonstrating intracellular Diels-Alder chemistry in live cells and to washout of HeLa cells more rapidly and completely than TCO and oxo-TCO analogs.

Acknowledgements

This work was supported by NIH GM132460 and Pfizer. Instrumentation was supported by NIH awards P20GM104316, P30GM110758, S10RR026962, and S10OD016267 and NSF awards CHE-0840401, CHE-1229234, and CHE-1048367. J. R. and A. J. were supported as CBI fellows through NIH T32-GM1333395.

= equal contributions

Keywords: *trans*-cyclooctene • tetrazine • diastereoselective • hydrophilic • bioorthogonal

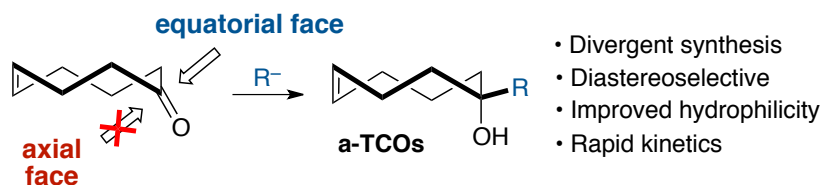
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