Studies on the Stability and Stabilization of *trans*-Cyclooctenes through Radical Inhibition and Silver (I) Metal Complexation

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Abstract. Conformationally strained *trans*-cyclooctenes (TCOs) engage in bioorthogonal reactions with tetrazines with second order rate constants that can exceed 10^6 $M^{-1}s^{-1}$. The goal of this study was to provide insight into the stability of TCO reagents and to develop methods for stabilizing TCO reagents for long-term storage. The radical inhibitor Trolox suppresses TCO isomerization under high thiol concentrations and TCO shelf-life can be greatly extended by protecting them as stable Ag(I) metal complexes. ^{1}H NMR studies show that Ag-complexation is thermodynamically favorable but the kinetics of dissociation are very rapid, and TCO•$AgNO_3$ complexes are immediately dissociated upon addition of NaCl which is present in high concentration in cell media. The $AgNO_3$ complex of a highly reactive s-TCO-TAMRA conjugate was shown to label a protein-tetrazine conjugate in live cells with faster kinetics and similar labeling yield relative to a 'traditional' TCO-TAMRA conjugate.

Keywords. bioorthogonal chemistry • *trans*-cyclooctene • stability • radical inhibitor • silver complexation • cellular labeling

Dedicated to Professor Stephen L. Buchwald in recognition of his contributions to the development of new reactions for chemical biology and on the occasion of the 2018 Tetrahedron Prize.

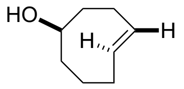
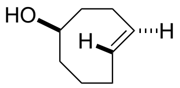
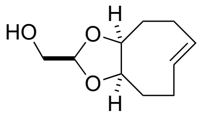
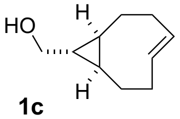
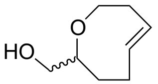
Introduction

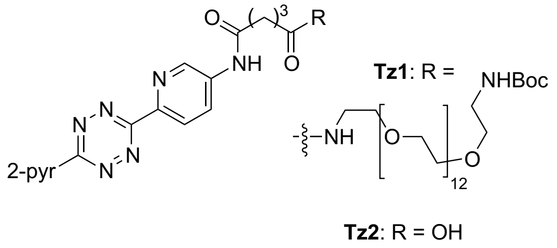
For nearly two decades, bioorthogonal chemistry has been employed for a broad range of applications spanning biomedicine and biotechnology. Tetrazine ligation— the cycloaddition of alkenes/alkynes and *s*-tetrazines—is a uniquely fast bioorthogonal reaction first developed in 2008 using *trans*-cyclooctene (TCO)[1] and norbornene dienophiles.[2] Tetrazine ligation since has become a broadly used tool for chemical biology, chemoproteomics, radiochemistry, and materials science.[3–12] A complementary set of dienophiles for tetrazine ligation has been developed that includes cyclopropenes, cyclobutenes, cyclooctynes and simple α-olefins.[13–22] However, *trans*-cyclooctene still maintains the advantage of exceptional reaction kinetics in this process, with reaction rates that exceed 10^4 $M^{-1}s^{-1}$ with parent TCO compounds.[1,23] The conformationally strained *trans*-cyclooctenes d-TCO (**1b**) and s-TCO (**1c**) display even faster reactivity, and participate in extremely rapid bioorthogonal reactions with tetrazines with rates exceeding 10^6 $M^{-1}s^{-1}$.[23,24] Recently, *trans*-oxocenes were introduced as dienophiles with improved aqueous solubility that maintain high reactivity due to the short C-O bond in the backbone.[25,26]

To choose the best chemical tool for a biological interrogation, it is important to understand the relative rates of the bioorthogonal reaction as well as the rates of competing side reactions. For some bioorthogonal reagents, side reactions are biomolecular and can result in non-selective background labeling. By contrast, the primary mechanism for the deactivation of TCO reagents is isomerization to the *cis*-isomer. For cellular applications, isomerization decreases labeling efficiency but has the merit of not resulting in non-selective off-target labeling.[1,23,24,26] Scheme 1 summarizes data from the literature, as well as newly collected data on the kinetic, stability and solubility characteristics of a series of *trans*-cyclooctene analogs. In the tetrazine ligation with **Tz1** or the closely related **Tz2**, d-TCO (**1b**), s-TCO (**1c**) and oxoTCO (**1d**) possess kinetic advantages over the parent TCO compound **1a**, but are also more prone to isomerization upon extended exposure to aqueous thiol concentrations that mimic the cellular environment.[23,24,26] Due to their propensity to isomerize, the more reactive TCOs **1b-1d** have greatest utility as probes for live cell applications, where only brief incubation in the cell is required due to the fast kinetics of the reaction. The use of TCOs as chemical reporters, which requires long-term stability in the cell, generally requires use of more resilient parent TCOs such as **1a**. An additional, practical consideration for strained TCO derivatives of **1b-d** is that they can be prone to deactivation upon long-term storage. While crystalline derivatives can be stored as solids over long periods (>1 year) in the refrigerator, non-solids must be stored as dilute solutions in the freezer and generally should be used within several weeks of preparation.

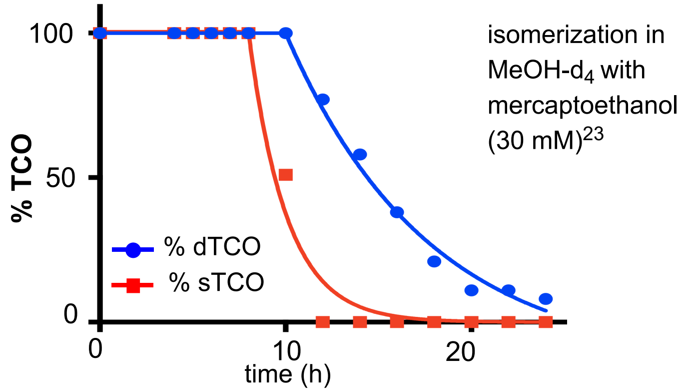
Scheme 1. (A) Kinetic and stability properties of *trans*-cyclooctene derivatives under varied conditions. (B) Isomerization kinetics of s-TCO and d-TCO in MeOH containing mercaptoethanol.

A

TCO structure	**Tetrazine kinetics** (k_2) 25 °C, H_2O	**TCO stability**
 **1a** (equatorial) logP 1.1[23]	22,600 $M^{-1}s^{-1}$ with **Tz1**[23]	• CD_3OD, 25 °C: >99% stable after 7 days[a] • PBS 37 °C, 'stable' after 7 days[30] • 10 mM glutathione in D_2O-PBS (pD 7.4) with 10 mM glutathione, 25 °C: 88% stable after 24 h[23] • PEGylated derivative in fresh mouse serum, $t_{1/2}$ 3.26 h[30] • PEGylated derivative, in PBS with human transcuprein (0.9 mg/mL), $t_{1/2}$ 1.39 h[30] • PEGylated derivative, in PBS with mouse serum albumin (21.7 mg/mL), $t_{1/2}$ 0.65 h[30] • *ax. isomer*: antibody conjugate, *in vivo* stability in mouse, $t_{1/2}$ 6.19 days[30]
 **1a'** (axial)	80,200 $M^{-1}s^{-1}$ with **Tz1**[23]	• CD_3OD, 25 °C: >99% stable after 7 days[a] • PEGylated derivative in fresh mouse serum, $t_{1/2}$ 3.36 h[30] • antibody conjugate, *in vivo* stability in mouse, $t_{1/2}$ 3.94 days[30]
 **1b** logP 0.95[23]	366,000 $M^{-1}s^{-1}$ with **Tz1**[23]	• CD_3OD, 25 °C: 95% stable after 3 days; 85% stable after 7 days[23] • D_2O-PBS (pD 7.4), 25 °C: no decomposition or isomerization after 14 days[23] • human serum: no decomposition or isomerization after 1 day; 97% stable after 4 days.[23] • 10% rabbit reticulocyte lysate in D_2O-PBS (pD 7.4): 12%, 22% and 41% isomerization after 10, 24, and 96 hours[23] • 10 mM glutathione in D_2O-PBS (pD 7.4), 25 °C: 53% isomerization after 4 hours, and 92% isomerization after 8 hours[23]
 **1c**	3,300,000 $M^{-1}s^{-1}$ with **Tz1**[23]	• in CD_3OD, 25 °C: 96% stable after 3 days[a] • D_2O-PBS (pD 7.4), 25 °C: 69% stable after 3 days[a] • 30 mM thiol (EtSH or 2-mercaptoethanol): compound is stable for 2-10 hours; isomerization takes place after this induction period (See Fig 1B below).[23,24]
 **1d** logP 0.51 (2.2:1 eq/ax mixture)[26]	eq. isomer 44,000 $M^{-1}s^{-1}$ with **Tz2** in PBS[26] ax. isomer 310,000 $M^{-1}s^{-1}$ with **Tz2** in PBS[26]	• CD_3OD, 25 °C: both diastereomers, >95% stable after 14 days[26] • D_2O-PBS (pD 7.4), 25 °C[26] *eq. isomer*: 90% stable after 7 days *ax. isomer*: $t_{1/2}$ 1.5 days • 25 mM 2-mercaptoethanol in D_2O-PBS (pD 7.4), 25 °C[26] *eq. isomer*: $t_{1/2}$ 2.2 h *ax. isomer*: $t_{1/2}$ 1.6 h



B

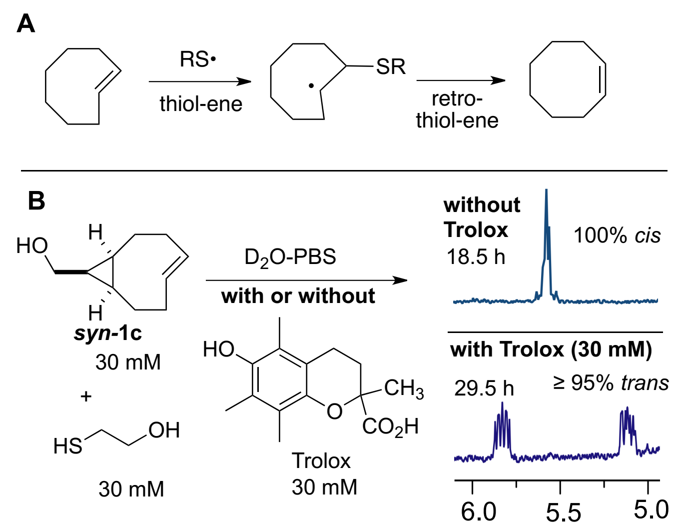


[a] Data collected in this study.

Results and Discussion

We have described previously that the isomerization of *trans*-cyclooctenes is promoted by high concentrations of thiols.[1,23,24,26] Thiol-promoted isomerization is observed across all of the TCOs **1a-d**, and in some cases is characterized by a long induction time before isomerization commences.[23] For example, we previously noted that solutions of d-TCO **1b** and s-TCO **1c** were stable in CD_3OD containing 30 mM mercaptoethanol for 12 and 8 hours, respectively, but subsequently underwent rapid isomerization (Scheme 1B).[23] It was hypothesized that the isomerization of d-TCO and s-TCO may be a radical catalyzed reaction, and at high thiol concentrations the reaction may be promoted by a thiol-ene/retro thiol-ene pathway (Scheme 2A).[27] A radical mechanism would be consistent with the unusual kinetic profile and induction period. During the induction period, the dissolved oxygen concentration is likely depleted through disulfide formation, and below a critical oxygen concentration the isomerization pathway would be favored. Radical intermediates have also been proposed to play an important role to the isomerization of *trans*-cycloheptenes through an 'interrupted dimerization' mechanism that follows second order kinetic behavior,[28,29] and may also explain the higher propensity of *trans*-cyclooctenes to isomerize or degrade as neat materials. Robillard and coworkers have demonstrated that Cu-containing proteins can promote TCO isomerization.[30] Cu-containing proteins are well known to engage in one-electron redox chemistry,[31] again suggesting the possible involvement of radical pathway for the isomerization of TCO.

The goal of this study was to provide additional evidence for radical intermediates in TCO isomerization and to develop methods for stabilizing TCO reagents for long-term storage. Initially, we sought to test if radical inhibitors could be used to suppress *trans*-cycloooctene isomerization under high thiol concentrations where isomerization is normally rapid. In a control experiment, a 30 mM solution of the *syn*-diastereomer of s-TCO (***syn*-1c**) and 30 mM mercaptoethanol were mixed in D_2O-PBS (pD 7.4) and isomerization was monitored by ^1H NMR after 1, 2, 4.5 and 18.5 hours, at which points 18%, 34%, 55% and 100% of the *cis*-isomer was detected, respectively. This isomerization was completely suppressed in a similar study where the radical inhibitor Trolox[32] – a water-soluble analog of vitamin E – was included in the mixture. Thus, treatment of ***syn*-1c** with 30 mM mercaptoethanol and 30 mM

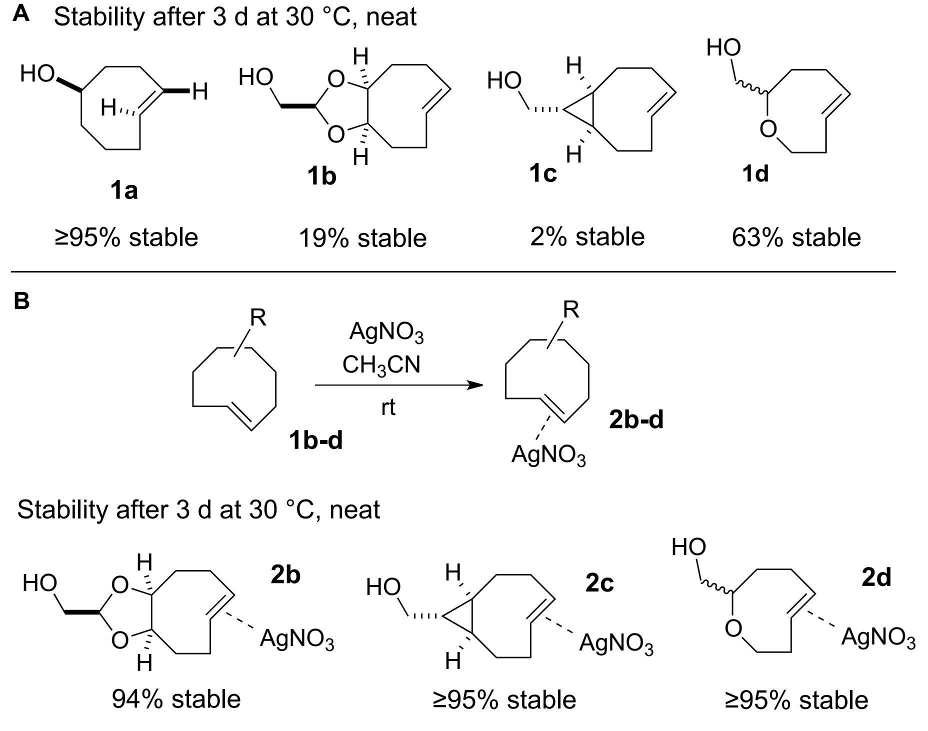


Scheme 2. (A) Plausible mechanism for the thiol-promoted isomerization of *trans*-cyclooctenes. (B) Isomerization of ***syn*-1c** is promoted by mercaptoethanol and inhibited by Trolox, a water soluble vitamin E analog.

Trolox in D_2O-PBS led to <5% isomerization (NMR detection limit) after 29.5 h (Scheme 2B and Fig S4). Similar observations were observed in isomerization studies of ***syn*-1c** in methanolic solutions containing BHT, a radical inhibitor that has been used to stabilize TCO.[33] Thus, complete isomerization was observed after 63 hours for a solution of 30 mM ***syn*-1c** in methanol containing 30 mM mercaptoethanol, whereas no isomerization was observed for a solution of 30 mM ***syn*-1c** and 30 mM mercaptoethanol containing 30 mM BHT (Fig S6). In the presence of low concentrations of a radical inhibitor, an induction period was observed prior to olefin isomerization. Thus, for 30 mM ***syn*-1c** in D_2O-PBS in the presence of 30 mM mercaptoethanol and 1 mM Trolox, <5% isomerization was observed after 6.5 hours, but 52% isomerization was observed after 21 hours (Fig S5). Taken together, these observations that thiols promote isomerization, that high concentrations of Trolox inhibit isomerization in the presence of thiol, and that an induction period is seen at low concentrations of Trolox all provide support for a radical based pathway for TCO-isomerization in the presence of thiols. Given the high intracellular concentrations of thiols and the role of thiols in cellular redox regulation,[34] it is also plausible that intracellular mechanisms for the deactivation of *trans*-cyclooctenes involve radical pathways.

Previously, we described that the shelf-life of conformationally strained *trans*-cycloalkene derivatives could be enhanced by protecting them as Ag(I) metal complexes, and that the free *trans*-cycloalkenes could be liberated by treatment with aq. NaCl or in cell media.[35] Metal complexation was shown to be essential for the handling of sila-*trans*-cycloheptene complexes,[29,36] which upon metal decomplexation participate in the fastest bioorthogonal reactions studied to date. Additionally, *trans*-cycloalkene $AgNO_3$ complexes possess high water solubility relative to the free alkenes, which can provide advantages for biological experiments. A goal of the present study was to establish the generality of Ag(I) complexation for the stabilization across a series of *trans*-cyclooctene derivatives. It is shown that Ag(I) metal complexation by *trans*-cyclooctenes is

Scheme 3. Stability comparisons for non-crystalline *trans*-cyclooctene derivatives when stored neat at 30 °C.



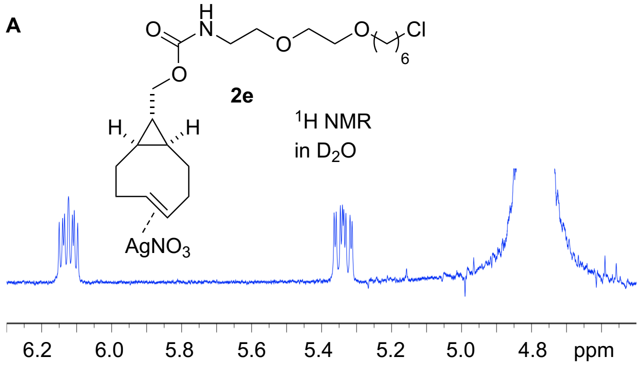
thermodynamically favorable but kinetically reversible, and that Ag(I) exchange between TCO ligands is rapid on the NMR timescale at low temperature. NMR studies also show that the kinetics of NaCl-mediated decomplexation are very rapid. Finally, it is shown that a s-TCO-TAMRA•$AgNO_3$ complex rapidly labels a protein-tetrazine conjugate in live cells with yields that are similar to a TAMRA-TCO conjugate but with rates that are significantly faster.

Shown in Scheme 3 is the comparison of the stabilities for a series of Ag-free TCO complexes and their $AgNO_3$ complexes after storage in an open flask for 3 days at 30 °C. By ^{1}H NMR analysis, the equatorial diastereomer **1a** is stable under these conditions, whereas the more reactive *trans*-cycloalkenes d-TCO **1b**, s-TCO **1c** and oxoTCO **1d** lead to 81%, 98% and 37% degradation. We note that these TCOs are much more stable in solution (Scheme 1), especially when stored in the freezer. For neat materials, undefined degradation products are formed, whereas olefin isomerization is observed in solution.

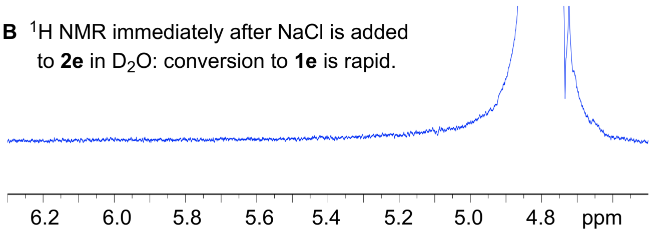
The preparation of *trans*-cyclooctene silver nitrate complex derivatives is straightforward and involves simply combining the TCO with $AgNO_3$ in acetonitrile or methanol for 15 min, followed by concentration under reduced pressure. When neat s-TCO•$AgNO_3$ (**2c**) was stored in an open flask at 30 °C for 3 days, the sample maintained ≥95% purity as determined by ^{1}H NMR with an internal standard. We also studied the stability of complex **2c** in methanol-d_4 and DMSO-d_6. After 3 days at 30 °C, the samples were still ≥95% pure. With d-TCO•$AgNO_3$ (**2b**), there was 94% fidelity for a neat sample that was stored in the open for 3 days at 30 °C, while in methanol and DMSO, d-TCO•$AgNO_3$ had 95% and 94% fidelity after 3 days at 30 °C respectively. oxoTCO•$AgNO_3$ (**2d**) showed ≥95% stability when stored neat at 30 °C for 3 days. In the freezer, the TCO•$AgNO_3$ complexes can be stored neat or in solution for >1 month without degradation.

We next conducted an NMR experiment that demonstrates that dissociation of a *trans*-cyclooctene silver complex by NaCl is very rapid (Scheme 4).

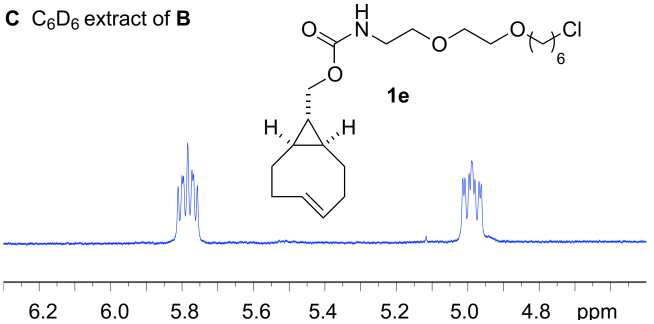
Scheme 4. NaCl decomplexation experiment



B ^{1}H NMR immediately after NaCl is added to **2e** in D_2O: conversion to **1e** is rapid.

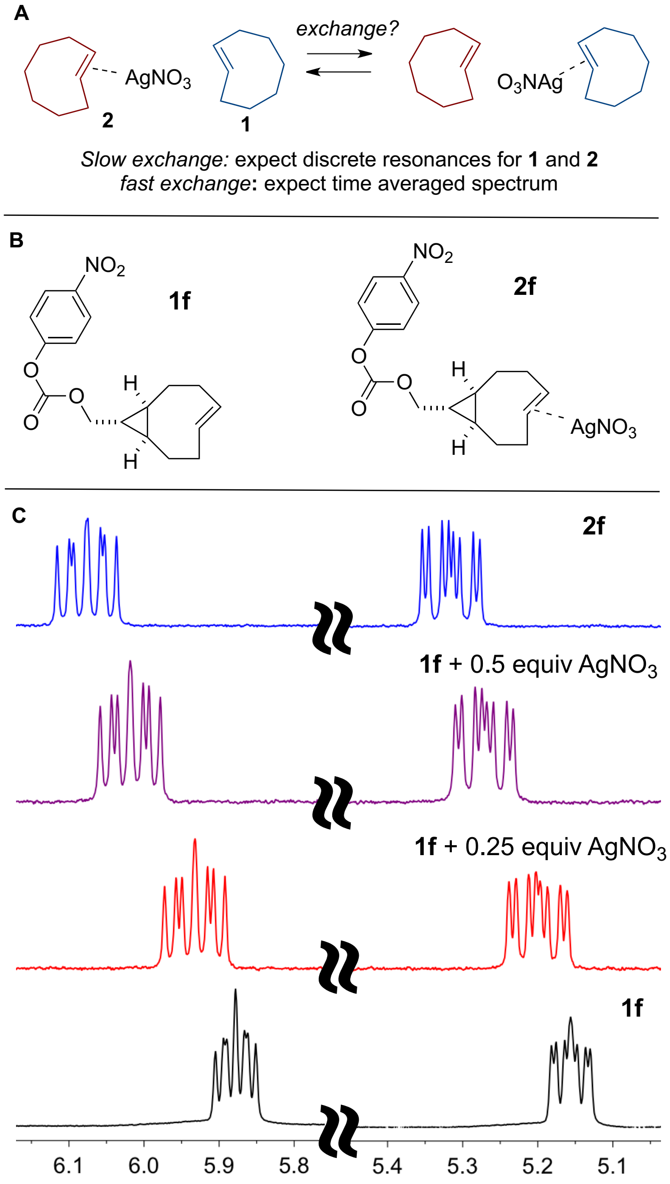


C C_6D_6 extract of **B**



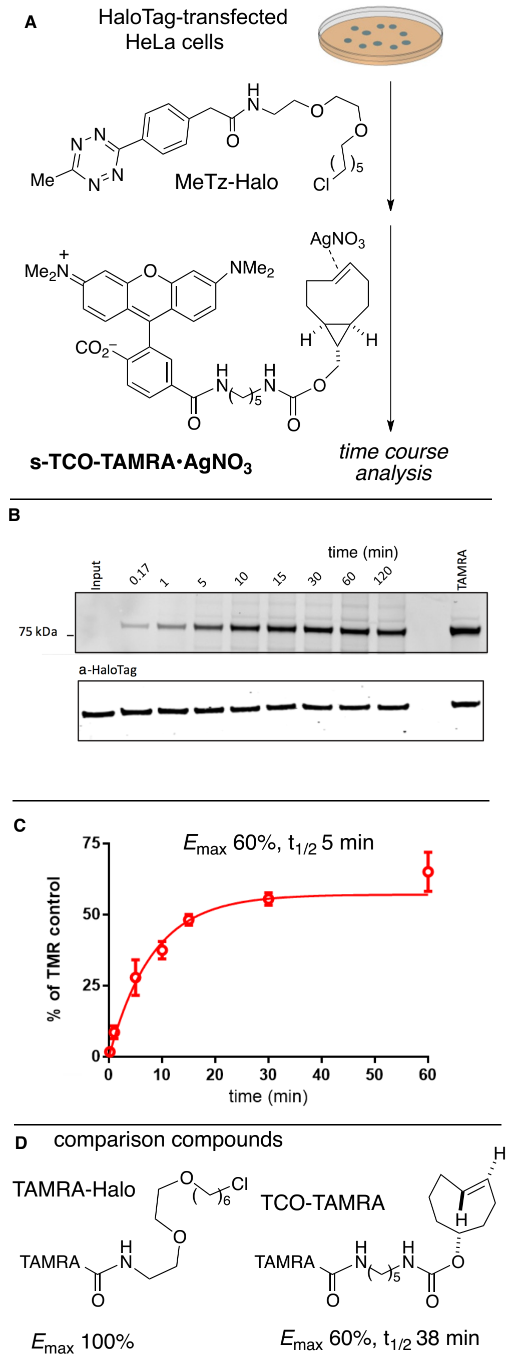
Here, compound **2e** (5 mg) was dissolved in D_2O (0.9 mL), followed by the addition of NaCl in D_2O (0.1 mL, 100 mM). Within 3 minutes, the ^{1}H NMR was remeasured, and no signal was observed. Upon extraction with C_6D_6, silver free **1e** was recovered and observed by ^{1}H NMR (Scheme 4). The experiment indicated that silver decomplexation of **2e** was complete by the time an ^{1}H NMR spectrum could be recorded.

Scheme 5 (A) Schematic depiction of metal exchange between TCO ligands and study by ^{1}H NMR spectroscopy. (B) Structures of TCO **1f** and TCO•$AgNO_3$ **2f** used for ligand exchange study. (C–F) ^{1}H NMR (CD_3OD, 600 MHz) spectra of alkene regions of (F) **1f,** (C) **2f,** and mixtures obtained by mixing **1f** with (E) 0.25 equiv $AgNO_3$ or (D) 0.5 equiv $AgNO_3$.



Slow exchange: expect discrete resonances for **1** and **2**
fast exchange: expect time averaged spectrum

^{1}H NMR spectroscopy was also used to study the kinetics and reversibility of Ag(I) coordination by TCO ligands. Previous x-ray crystallography studies of *trans*-cycloalkene silver complexes show that the alkene coordinates to the metal with 1:1 stoichiometry.[29,37] We queried whether the exchange of a TCO ligand **1** with a TCO•$AgNO_3$ complex **2** would be slow enough to observe by ^{1}H NMR spectroscopy. If exchange is slow on the NMR timescale, discrete resonances would be expected for metal complex **2** and free alkene **1**, whereas rapid exchange would lead to a time averaged spectrum of **1** and **2** (Scheme 5A). Combining TCO **1f** with 1 equivalent $AgNO_3$ to give metal complex **2f** was accompanied by downfield shifts for the alkene resonances in the ^{1}H NMR spectrum. Adding additional (0.5 equivalent) $AgNO_3$ did not lead to further change in the NMR spectrum, consistent with 1:1 metal:alkene stoichiometry for the solution complex. As shown in Scheme 5, when fewer than 1.0 equivalent of $AgNO_3$ was added to alkene **1f**, time averaged NMR spectra were observed with a downfield shift proportional to the amount of $AgNO_3$ that was added. Thus, for the alkene peak at 5.87 ppm, the addition of 0.25 and 0.5 equivalent of $AgNO_3$ caused downfield shifts of ~0.06 and 0.12 ppm, respectively. The ^{1}H NMR of **1f** with 0.5 equivalent of $AgNO_3$ was also studied by VT-NMR down to –60 ° C, and coalescence was not observed, indicating that exchange



Scheme 6. (A) Incorporation of MeTz-Halo into HaloTag-transfected HeLa cells, followed by labeling with s-TCO-TAMRA•AgNO$_3$ (2 μM). (B,C) Kinetics of labeling were evaluated by timecourse quenching with a non-fluorescent TCO with subsequent analysis by in-gel fluorescence. (D) The degree of labeling was benchmarked against cells in which TAMRA-Halo was directly incorporated, and cells in which MeTz-Halo incorporation was followed by labeling with TCO-TAMRA.

remains rapid on the NMR timescale at this temperature. Thus, while Ag-complexation is thermodynamically favorable for *trans*-cyclooctene, the complexes are labile in aqueous NaCl and the kinetics of dissociation are very rapid. Similar experiments were also carried out with the AgNO$_3$ complex of **1a** (Fig S8-9). For a 2:1 mixture of **1a**: AgNO$_3$, ligand exchange is rapid on the ^{1}H NMR timescale at room temperature, but the NMR spectrum is broadened at –60 °C suggesting a faster rate of exchange for this less strained TCO.

The high reactivity of conformationally strained *trans*-cycloalkenes make them useful probe compounds for applications where brief incubation in the cellular environment is required.[29,35,38] In a previous study, the HaloTag protein-labeling platform was used to compare the rates and yields of various bioorthogonal reactions in live cells.[35] Here, we surveyed a range of different HaloTag ligands containing bioorthogonal tags, including 3-methyl-6-phenyl-s-tetrazine. Pulse-chase experiments with a TAMRA-labeled chloroalkane control (TAMRA-Halo) showed that a similar percentage of incorporation was observed after a 30 min incubation for all of the HaloTag ligands in that series (70-79%), allowing for subsequent comparisons that would reflect differences in the labeling efficiency.

We also utilized silver complexes of s-TCO and d-TCO HaloTag conjugates to tag proteins with subsequent time course labeling by fluorescent tetrazine derivatives.[35] Here, labeling efficiency decreases after prolonged cellular incubation, presumably due to TCO inactivation. We expected that reversing the tag and probe and use of a fluorescent s-TCO conjugate would enable more efficient labeling. To this end, an s-TCO-TAMRA•$AgNO_3$ conjugate was prepared by mixing s-TCO-TAMRA with silver nitrate. Metal complexation resulted in a characteristic downfield shift of the alkene peaks in the ^1H NMR spectrum, and it was confirmed that mixing s-TCO-TAMRA•$AgNO_3$ with aq. NaCl rapidly resulted in metal decomplexation to regenerate s-TCO-TAMRA (Fig S2). Our previously reported haloalkane dehalogenase "HaloTag"-based platform was used to study the tetrazine ligation in live cells using s-TCO-TAMRA•$AgNO_3$. In a cellular co-localization experiment, it was first confirmed that the TAMRA signal co-localizes with HaloTag-H2B-GFP fluorescence when live HeLa cells were labeled with 500 nM of s-TCO-TAMRA (Figure S7). To study the kinetics of labeling, HaloTag-transfected HeLa cells were then treated with MeTz-Halo (10 μM), washed and then treated with s-TCO-TAMRA•$AgNO_3$ (2 μM) for different incubation times up to 60 min (Scheme 6). Both the concentration and exposure time of $AgNO_3$ to live cells are much lower than that used in cytotoxicity studies on HeLa cells (IC50 = 50 μM after 72 h).[41] The tetrazine attached to HaloTag-H2B-GFP was quenched at the various time points by chasing with excess non-fluorescent TCO and in-gel fluorescence was used to quantify conversion vs. time. The labeling efficiency of s-TCO-TAMRA•$AgNO_3$ was compared to a TAMRA-labeled chloroalkane control (TAMRA-Halo, Promega Corporation) that was directly incorporated into HaloTag-H2B-GFP transfected HeLa cells, and also to two-step labeling with MeTz-Halo and TCO-TAMRA cells (Scheme 6D).

As shown in Scheme 6B,C, saturation was reached rapidly with a labeling $t_{1/2}$ of 5 min and with 60% of the fluorescence intensity obtained with TAMRA-Halo.[35] The maximum fluorescence of the two-step procedures involving tetrazine ligation was 60% of that obtained when TAMRA-Halo was directly incorporated. The lower labeling yields for the two-step procedures is partly due to a lower incorporation yield with MeTz-Halo relative to TAMRA-Halo[35] and may be partly due to inactivation of MeTz in the intracellular environment. As shown in Figure 6, the 60% relative fluorescence with s-TCO-TAMRA•$AgNO_3$ (**3**) was similar to that observed when a TAMRA conjugate of 5-hydroxy-*trans*-cyclooctene (TCO-TAMRA, Scheme 6D) was used,[35] but the time to reach saturation was much slower ($t_{1/2}$ = 38 min) with the 5-hydroxy-*trans*-cyclooctene conjugate.

Conclusions

In summary, this study shows how the lifetime of conformationally strained *trans*-cyclooctenes can be extended through the addition of radical inhibitors or through silver (I) metal complexation. The radical inhibitor Trolox is shown to suppress *trans*-cycloooctene isomerization under high thiol concentrations where isomerization is normally rapid, thereby providing support for a radical-mediated mechanism for TCO-isomerization. Additionally, the shelf-life of conformationally strained *trans*-cyclooctenes can be greatly extended by protecting them as stable Ag(I) metal complexes. VT-NMR studies show that Ag-complexation is thermodynamically favorable for *trans*-cyclooctene, but the complexes are labile and the kinetics of dissociation are very rapid. Further NMR studies show that TCO•$AgNO_3$ complexes are immediately dissociated upon addition of NaCl. Thus, the most highly strained *trans*-cyclooctenes can be stabilized for long term storage through Ag(I) complexation, and then liberated on demand by addition of NaCl which is present in high concentration in cell media. To illustrate the utility of silver complexation in cellular labeling experiments, the silver nitrate complex of a highly reactive s-TCO-TAMRA conjugate was prepared, and was shown to label a protein-tetrazine conjugate in live cells with faster kinetics and similar labeling yield relative to an 'ordinary' TCO-TAMRA conjugate.

Experimental

General considerations. Anhydrous methylene chloride was dried through a column of alumina using a solvent purification system. Anhydrous THF was freshly distilled from Na/benzophenone ketyl. All reagents were purchased from commercial sources and used without further purification. Chromatography was performed on normal-phase silica gel from Silicycle (40-63 µm, 230-400 mesh). Reverse phase chromatography was performed on Teledyne Isco (Combiflash® RF). Conversions of photoisomerization reactions were monitored by GC (GC-2010 Plus, Shimadzu). APT and CPD pulse sequences were used for ^{13}C NMR. When the APT pulse sequence was used for ^{13}C NMR, the secondary and quaternary carbons were phased to appear 'up' (u), and tertiary and primary carbons appear 'down' (dn). Data are represented as follows: chemical shift, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets). High resolution mass spectral data were taken with a Waters GCT Premier high-resolution time-of-flight mass spectrometer, or using a Thermo Q-Exactive Orbitrap instrument. Infrared (IR) spectra of compound **2c** was obtained using FTIR spectrophotometers with films cast onto AgCl plate. Infrared (IR) spectra for compound **2c**, **2b**, **2f** were collected from Bruker Tensor 27 and Nexus^{TM} 670 FT-IR. ((1R,8S,9r,E)-bicyclo[6.1.0]non-4-en-9-yl)methanol (s-TCO)[24], ((1R,8S,9r,E)-bicyclo[6.1.0]non-4-en-9-yl)methyl (4-nitrophenyl) carbonate (s-TCO-NO_2)[24], ((2r,3aR,9aS,E)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol (d-TCO)[23], ((1R,8S,9s,E)-bicyclo[6.1.0]non-4-en-9-yl)methyl (2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamate•$AgNO_3$[34], (*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol[26] (oxoTCO) and 5-((3-aminopropyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate[39] were prepared following known procedures.

Synthesis of *trans*-Cyclooctene Silver Nitrate Complex Derivatives

((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol•$AgNO_3$ (s-TCO•$AgNO_3$) (2c). To a flask containing ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol (16.1 mg, 0.106 mmol, 1.00 equiv) was added an acetonitrile (1.91 ml, 10.3 mg/ml) solution of silver nitrate (19.8 mg, 0.116 mmol, 1.10 equiv). The reaction mixture was stirred at room temperature for 15 min. The mixture was concentrated under reduced pressure to afford (37.6 mg, quantitative yield) as white semisolid. ^{1}H NMR (400 MHz, CD_3OD) δ: 6.10-6.03 (m, 1H), 5.28 (ddd, *J* =16.6, 10.6, 3.5Hz, 1H), 3.49-3.41 (m, 2H), 2.55-2.44 (m, 2H), 2.35-2.29 (m, 1H), 2.25-2.20 (m, 2H), 1.98-1.88 (m, 1H), 1.08-0.97 (m, 1H) , 0.87-0.77 (m, 1H), 0.67-0.60 (m, 1H), 0.49-0.41(m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ: 126.2 (dn), 118.8 (dn), 66.9 (u), 38.7 (u), 35.2 (u), 33.0 (u), 29.26 (dn), 29.25 (u), 22.2 (dn), 20.7 (dn); FTIR (ATR) 3436, 3003, 2978, 2914, 2850, 1572, 1448, 1385, 1300, 1111, 1076, 1041, 1014, 985, 928, 891, 843 cm^{-1}; HRMS (CI+) m/z: $[M-AgNO_3+H]^+$ calcd. for $C_{10}H_{17}O^+$ 153.1274, found 153.1272.

((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol•$AgNO_3$ (d-TCO•$AgNO_3$) (2b). To a flask containing ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol

(15.6 mg, 0.0847 mmol, 1.00 equiv) was added an acetonitrile (1.53 ml, 10.3 mg/ml) solution of silver nitrate (15.8 mg, 0.0931 mmol, 1.10 equiv). The reaction mixture was stirred at room temperature for 15 min. The mixture was concentrated under reduced pressure to afford (33.8 mg, quantitative yield) as white semisolid. ^{1}H NMR (600 MHz, CD_3CN) δ: 5.71-5.57 (m, 2H), 4.71 (t, *J* =3.7 Hz, 1H), 3.97 (d, *J* =9.9 Hz, 2H), 3.48-3.42 (m, 2H), 3.39-3.26 (m, 1H), 2.38-2.35 (m, 1H), 2.33-2.28 (m, 1H), 2.12-2.05 (m, 3H) , 1.76-1.56 (m, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ: FTIR (ATR) 3395, 2934, 2866, 1576, 1396, 1140, 1034, 997, 903, 856, 818 cm^{-1}; HRMS (CI+) *m/z*: $[M\text{-}AgNO_3\text{+}H]^+$ calcd. for $C_{10}H_{17}O_3^+$ 185.1172, found 185.1179.

(*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol•AgNO$_3$ (oxoTCO•AgNO$_3$) (2d). To a flask containing (*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol (11.6 mg, 0.0816 mmol, 1.00 equiv) was added an acetonitrile (89.7 µL, 1.0 M) solution of silver nitrate (15.2 mg, 0.0897 mmol, 1.10 equiv). The reaction mixture was stirred at room temperature for 1 hour. The mixture was concentrated under reduced pressure to afford (*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol•AgNO$_3$ (28.0 mg, quantitative yield) as colorless semisolid.

Peaks due to major diastereomer: ^{1}H NMR (400 MHz, CD_3OD) δ: 5.87 (ddd, *J* = 15.8, 11.4, 2.9 Hz, 1H), 5.57 (ddd, *J* = 15.7, 11.0, 3.3 Hz, 1H), 4.14-4.09 (m, 1H), 3.48-3.41 (m, 3H), 3.14-3.08 (m, 1H), 2.72-2.67 (m, 1H), 2.54-2.49 (m, 1H), 2.31-2.13 (m, 2H), 2.02-1.81 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ: 129.4 (dn), 114.8 (dn), 86.7 (dn), 74.5 (u), 66.3 (u), 39.3 (u), 38.3 (u), 35.1 (u);

Peaks due to minor diastereomer: ^{1}H NMR (400 MHz, CD_3OD) δ: 6.05-5.97 (m, 1H), 5.55-5.51 (m, 1H), 3.91-3.85 (m, 1H), 3.84-3.79 (m, 2H), 3.68-3.62 (m, 1H), 3.47-3.42 (m, 1H), 2.56-2.50 (m, 1H), 2.45-2.44 (m, 2H), 2.31-2.13 (m, 1H), 2.01-1.83 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ: 126.7 (dn), 117.3 (dn), 81.9 (dn), 68.8 (u), 64.2 (u), 39.5 (u), 36.6 (u), 30.0 (u); FTIR (AgCl film) 3408, 3006, 2942, 2840, 1658, 1607, 1482, 1302, 1210, 1197, 1159, 1096, 1063, 1035, 944, 883 cm^{-1}; HRMS (ESI+) *m/z*: $[M\text{+}H\text{-}AgNO_3]^+$ calcd. for $C_8H_{15}O_2^+$ 143.1067, found 143.1067.

((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methyl (4-nitrophenyl) carbonate•$AgNO_3$ (2f). To a flask containing ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methyl (4-nitrophenyl) carbonate (31.7 mg, 0.0999 mmol, 1.00 equiv) was added an acetonitrile (0.813 ml, 22.96 mg/ml) solution of silver nitrate (18.7 mg, 1.10 mmol, 1.10 equiv). The reaction mixture was stirred at room temperature for 1 hour. The mixture was concentrated under reduced pressure to afford (51.5 mg, quantitative yield) as white semisolid. ^1H NMR (600 MHz, CD_3OD) δ: 8.31 (d, *J* =9.1 Hz, 2H), 7.46 (d, *J* = 9.1 Hz, 2H), 6.07 (ddd, *J* =16.5, 8.9, 6.2 Hz, 1H), 5.31 (ddd, *J* =16.5, 10.6, 3.6 Hz, 1H), 4.23-4.18 (m, 2H), 2.57-2.54 (m, 1H), 2.51-2.47 (m, 1H), 2.36-2.33 (m, 1H), 2.30-2.22 (m, 2H), 2.00-1.93 (m, 1H), 1.12-1.05 (m, 1H), 0.92-0.82 (m, 2H), 0.72-0.64 (m, 2H); ^{13}C NMR (150MHz, CD_3OD) δ: 157.2 (u), 154.1 (u), 146.8 (u), 126.2 (dn), 125.8 (dn), 123.2 (dn), 118.7 (dn), 74.5 (u), 38.5(u), 35.0 (u), 32.8 (u), 29.1 (u), 25.5 (dn), 22.9 (dn), 21.4 (dn); FTIR (ATR) 2961, 2935, 1754, 1592,1513, 1442, 1344, 1271, 1248, 1206, 1113, 1008, 922, 846, 750 cm^{-1}; HRMS (CI+) *m/z*: $[M-AgNO_3+H]^+$ calcd. for $C_{17}H_{20}NO_5^+$ 318.1336, found 318.1347.

5-((3-(((((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methoxy)carbonyl)amino)propyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate (s-TCO-TAMRA). To a DMSO (0.200 ml, anhydrous) solution of 5-((3-aminopropyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate TFA salt (10.0 mg, 0.0166 mmol, 1.00 equiv) and N, N-Diisopropylethylamine (8.68μL, 0.0498 mmol, 3.00 equiv) was added ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methyl (4-nitrophenyl) carbonate (6.34 mg, 0.0199 mmol, 1.20 equiv). The reaction mixture was stirred at room temperature for 1 hour. The mixture was directly loaded onto C18 column (Universal ™ Column, Yamazen Corporation, Cat.No. UW112, 14g) using minimal amount of acetonitrile followed by H_2O. The purification was performed by Teledyne Isco (Combiflash® RF), mobile phase A: 0.03% NH_3▪H_2O in water (v/v), mobile phase B: 0.03% NH_3▪H_2O in acetonitrile (v/v), 100% H_2O linear to 50% H_2O/50% acetonitrile, hold at 50% H_2O/50% acetonitrile for 10 min, with 10 ml/min flow rate. The mixture was then lyophilized to yield 5-((3-(((((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methoxy)carbonyl)amino)propyl)carbamoyl)-2-(6-(dimethylamino)-3-

(dimethyliminio)-3H-xanthen-9-yl)benzoate (s-TCO-TAMRA) (8.7 mg, 78%) as a dark red solid. ^{1}H NMR (400MHz, CD_3CN) δ: 8.34 (s, 1H), 8.13 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.60 (s, 1H), 7.23 (d, *J* = 8.1 Hz, 1H), 6.58 (d, *J* = 8.8 Hz, 2H), 6.49-6.44 (m, 4H), 5.86-5.75 (m, 2H), 5.10 (ddd, *J* = 16.9, 10.5, 3.9 Hz, 1H), 3.88 (d, *J* = 6.5 Hz, 2H), 3.45-3.40 (m, 2H), 3.20-3.15 (m, 2H), 2.96 (s, 12H), 2.31-2.11 (m, 4H), 1.91-1.82 (m, 2H), 1.75-1.69 (m, 2H), 0.92-0.80 (m, 1H), 0.62-0.50 (m, 2H), 0.44-0.36 (m, 2H);^{13}C NMR (100 MHz, CD_3CN) δ: 169.7 (u), 166.6 (u), 158.1 (u), 156.0 (u), 153.7 (u), 153.5 (u), 139.1 (dn), 137.4 (u), 134.8 (dn), 132.0 (dn), 129.6 (dn), 128.8 (u), 125.2 (dn), 124.2 (dn), 109.9 (dn), 107.0 (u), 99.0 (dn), 69.8 (u), 40.4 (dn), 39.3 (u), 38.6 (u), 37.6 (u), 34.4 (u), 33.2 (u), 30.4 (u), 28.3 (u), 25.7 (dn), 22.7 (dn), 21.7 (dn); HRMS (ESI+) *m/z*: $[M+H]^+$ calcd. for $C_{39}H_{45}N_4O_6^+$ 665.3334, found 665.3330.

5-((3-(((((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methoxy)carbonyl)amino)propyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate•$AgNO_3$ (s-TCO-TAMRA•$AgNO_3$). To a flask containing 5-((3-(((((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methoxy)carbonyl)amino)propyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate (9.20 mg, 0.0138 mmol, 1.00 equiv) in 1.0 ml methanol was added an acetonitrile (15.2 µL, 1.0 M) solution of silver nitrate (2.59 mg, 0.0152 mmol, 1.10 equiv). The reaction mixture was stirred at room temperature for 15 min. The mixture was concentrated under reduced pressure to afford 5-((3-(((((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methoxy)carbonyl)amino)propyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate•$AgNO_3$ (13.0 mg, quantitative yield) as red solid. ^{1}H NMR (400 MHz, CD_3CN) δ: 8.52 (s, 1H), 8.11-8.07 (m, 2H), 7.24 (d, *J* = 7.9 Hz, 1H), 6.92 (d, *J* = 9.2 Hz, 2H), 6.74 (dd, *J* = 9.2, 2.5 Hz, 2H), 6.69 (d, *J* = 2.5 Hz, 2H), 6.06(s, 1H), 5.91 (dt, *J* = 15.8, 7.5 Hz, 1H), 5.17-5.10 (m, 1H), 3.87 (d, *J* = 6.1 Hz, 2H), 3.44-3.38 (m, 2H), 3.16-3.12 (m, 14H), 2.40-2.32 (m, 2H), 2.21-2.17(m, 1H), 2.12-2.07 (m, 2H), 1.86-1.80 (m, 1H), 1.77-1.71 (m, 2H), 1.02-0.90 (m, 1H), 0.76-0.61 (m, 2H), 0.50-0.44(m, 2H); HRMS (ESI+) *m/z*: $[M+H-AgNO_3]^+$ calcd. for $C_{39}H_{45}N_4O_6^+$ 665.3334, found 665.3331.

Stability Properties of *trans*-Cyclooctenes

Stability study of *rel*-(1R, 4*E*, pR)-Cyclooct-4-enol (1a major diastereomer) in methanol. *rel*-(1R, 4*E*, pR)-Cyclooct-4-enol (22.0 mg) was mixed with 1,3,5-trimethoxybenzene (9.00 mg). The mixture was dissolved in 1.20 ml CD_3OD and ^{1}HNMR was taken as time = 0. The sample was stored at 25°C. ^{1}HNMR spectrum was taken after 3 days and 7 days. Overall there was >99% fidelity for *rel*-(1R, 4*E*, pR)-cyclooct-4-enol (5OH-TCO major diastereomer) after 3 days and >99% fidelity after 7 days.

Stability study of *rel*-(1R, 4*E*, pS)-Cyclooct-4-enol (1a minor diastereomer) in methanol. *rel*-(1R, 4*E*, pS)-Cyclooct-4-enol (11.0 mg) was mixed with 1,3,5-trimethoxybenzene (5.00 mg). The mixture was dissolved in 0.60 ml CD_3OD and ^{1}HNMR was taken as time = 0. The sample was stored at 25°C. ^{1}HNMR spectrum was taken after 3 days and 7 days. Overall there was >99% fidelity for *rel*-(1R, 4*E*, pS)-cyclooct-4-enol (5OH-TCO minor diastereomer) after 3 days and >99% fidelity after 7 days.

Stability test of ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol (s-TCO) in methanol. ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol (6.10 mg) was mixed with 1,3,5-trimethoxybenzene (4.00 mg). The mixture was dissolved in 0.7 ml CD_3OD and ^{1}HNMR was taken as time = 0. The sample was stored at 25°C. ^{1}HNMR spectrum was taken after 3 days and 7 days. Overall there was 96% fidelity for ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol (s-TCO) after 3 days and 86% fidelity after 7 days.

Stability test of ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol(s-TCO) in phosphate buffered D_2O (pD = 7.4). Phosphate D_2O buffer (pD 7.4) was prepared by dissolving sodium dihydrogen phosphate hydrate ($NaH_2PO_4 \cdot H_2O$, 19.3 mg) and disodium hydrogen phosphate (Na_2HPO_4, 51 mg) in 5 mL D_2O to make a 0.1 M solution, and then adjusting to pD 7.4 by adding DCl. The pD values were measured on an ATI PerpHect LogR pH meter (model 310). pH readings were converted to pD by adding 0.4 units.[40]

To a mixture of s-TCO (6.14 mg, 0.017 mmol) and phosphate buffered D_2O (1 mL, pD = 7.4) was added 4-methoxybenzoic acid (5.59 mg, 0.018 mmol) as an internal standard and monitored by ^{1} HNMR to observe the stability of s-TCO. Overall there was 69% fidelity for ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol (s-TCO) after 3 days at 25°C.

Stability comparisons for non-crystalline *trans*-cyclooctene derivatives when stored neat at 30 °C.

Neat stability test of *rel*-(1R, 4*E*, pR)-Cyclooct-4-enol (1a major diastereomer). *rel*-(1R, 4*E*, pR)-Cyclooct-4-enol (34.51 mg) was dissolved in acetone (3 ml). 1 ml of this acetone solution of *rel*-(1R, 4E, pR)-cyclooct-4-enol was aliquoted and acetone was dried under reduced pressure, after which 6.06 mg 1,3,5-trimethoxybenzene was added and ^{1}HNMR was taken in $CDCl_3$. The amount of *rel*-(1R, 4*E*, pR)-cyclooct-4-enol was determined by ^{1}HNMR as 0.0851 mmol. At the meantime, another 1ml of this acetone solution was dried under reduced pressure. This neat sample of *rel*-(1R, 4*E*, pR)-cyclooct-4-enol was heated at 30°C in an oil bath over 3 days. After that, this sample was mixed with 4.79 mg 1,3,5-trimethoxybenzene and ^{1}HNMR was taken in $CDCl_3$. The amount of *rel*-(1R, 4*E*, pR)-cyclooct-4-enol was determined as 0.0807 mmol. Overall, there was 95% fidelity for *rel*-(1R, 4*E*, pR)-cyclooct-4-enol after 3 days.

Neat stability test of *rel*-(1R, 4*E*, pS)-Cyclooct-4-enol (1a minor diastereomer). *rel*-(1R, 4*E*, pS)-Cyclooct-4-enol (39.8 mg) was dissolved in acetone (3 ml). 1 ml of this acetone solution of *rel*-(1R, 4*E*, pS)-Cyclooct-4-enol was aliquoted and acetone was dried under reduced pressure, after which 4.49 mg 1,3,5-trimethoxybenzene was added and ^{1}HNMR was taken in $CDCl_3$. The amount of *rel*-(1R, 4*E*, pS)-Cyclooct-4-enol was determined by ^{1}HNMR as 0.0942 mmol. At the meantime, another 1ml of this acetone solution was dried under reduced pressure. This neat sample of *rel*-(1R, 4*E*, pS)-Cyclooct-4-enol was heated at 30°C in an oil bath over 3 days. After that, this sample was mixed with 4.63 mg 1,3,5-trimethoxybenzene and ^{1}HNMR was taken in $CDCl_3$. The amount of *rel*-(1R, 4*E*, pS)-Cyclooct-4-enol was determined as 0.0923 mmol. Overall, there was 98% fidelity for *rel*-(1R, 4*E*, pS)-Cyclooct-4-enol after 3 days.

Neat stability test of ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol (d-TCO). ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol (15.0 mg) was dissolved in diethyl ether (3 ml). 1 ml of this ether solution of ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol was dried under reduced pressure and then mixed with 4.30 mg 1,3,5-trimethoxybenzene and 1HNMR was taken in $CDCl_3$. The amount of ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol in 1 ml diethyl ether was determined by ^{1}H NMR as 0.0376 mmol. At the meantime, another 1ml of this diethyl ether solution was dried under reduced pressure. This neat sample of ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol was heated at 30°C in an oil bath over 3 days. After that, this sample was mixed with 4.20 mg 1,3,5-trimethoxybenzene and ^{1}HNMR was taken in $CDCl_3$. The amount of ((2r,3aR,9aS, E)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol was determined as 0.00705 mmol. Overall, there was 19% fidelity for ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol after 3 days.

Neat stability test of ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol (s-TCO). ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol (20.0 mg) was dissolved in diethyl ether (3 ml). 1 ml of this ether solution of ((1R,8S,9r,*E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol was dried under reduced pressure and then mixed with 5.30 mg 1,3,5-trimethoxybenzene and ^{1}HNMR was taken in C_6D_6. The amount of ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol in 1 ml diethyl ether was determined by ^{1}HNMR as 0.0409 mmol. At the meantime, another 1ml of this diethyl ether solution was dried under reduced pressure. This neat sample of ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol was heated at 30°C in an oil bath over 3 days. After that, this sample was mixed with 5.10 mg 1,3,5-trimethoxybenzene and ^{1}HNMR was taken in C_6D_6. The amount of ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol was determined as 0.000705 mmol. Overall, there was 2% fidelity ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol (s-TCO) after 3 days.

Neat stability test of (*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol (oxoTCO). (*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol (11.6 mg) was dissolved in methanol (3 ml). 1 ml of this methanol solution of (*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol was aliquoted and methanol was dried under reduced pressure, after which 2.90 mg 1,3,5-trimethoxybenzene was added and ^{1}H NMR was taken in CD_3CN. The amount of

(*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol in 1 ml methanol was determined by ^{1}H NMR as 0.0187 mmol. At the meantime, another 1ml of this methanol solution was dried under reduced pressure. This neat sample of (*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol was heated at 30°C in an oil bath over 3 days. After that, this sample was mixed with 2.80 mg 1,3,5-trimethoxybenzene and ^{1}H NMR was taken in CD_3CN. The amount of (*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol was determined as 0.012 mmol. Overall, there was 37% decomposition for (*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol after 3 days.

Neat stability test of ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol•$AgNO_3$ (s-TCO•$AgNO_3$). ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol•$AgNO_3$ (57.7 mg) was dissolved in CH_3CN (3 ml). 1 ml of this CH_3CN solution of ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol•$AgNO_3$ was mixed with 4.26 mg 1,3,5-trimethoxybenzene, concentrated by rotatory evaporation and ^{1}H NMR was taken in CD_3CN (as time 0). The amount of ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol•$AgNO_3$ in 1 ml CH_3CN was determined by ^{1}H NMR (CD_3CN) as 0.0561 mmol. At the meantime, another 1ml of this CH_3CN solution was dried under reduced pressure. This neat sample of ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol•$AgNO_3$ was heated at 30°C in an oil bath over 3 days. After that, this sample was mixed with 6.20 mg 1,3,5-trimethoxybenzene and ^{1}H NMR was taken in CD_3CN. The amount of ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol•$AgNO_3$ was determined as 0.0543 mmol. Overall, there was 97% fidelity for a neat sample of ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol•$AgNO_3$ after 3 days.

Neat stability test of ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol •$AgNO_3$ (d-TCO•$AgNO_3$). ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol •$AgNO_3$ (33.8 mg) was dissolved in CH_3CN (3 ml). 1 ml of this CH_3CN solution of ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol •$AgNO_3$ was dried and mixed with 4.40 mg 1,3,5-trimethoxybenzene , then ^{1}HNMR was taken in CD_3CN. The amount of ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol •$AgNO_3$ in 1 ml CH_3CN was determined by ^{1}HNMR as 0.0283 mmol. At the meantime, another 1ml of this CH_3CN solution was dried under reduced pressure. This neat sample of ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol •$AgNO_3$ was heated at 30°C in an oil bath over 3 days. After that, this sample was mixed with 3.60 mg 1,3,5-trimethoxybenzene and ^{1}HNMR was taken in CD_3CN. The amount of ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol•$AgNO_3$ was determined as 0.0267 mmol. Overall, there was 94% fidelity for ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol•$AgNO_3$ after 3 days.

Neat stability test of (*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol•$AgNO_3$ (oxoTCO•$AgNO_3$). (*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol•$AgNO_3$ (28 mg) was dissolved in methanol (3 ml). 1 ml of this methanol solution of (*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol•$AgNO_3$ was aliquoted and methanol was dried under reduced pressure, after which 2.50 mg 1,3,5-trimethoxybenzene was added and ^{1}HNMR was taken in CD_3CN. The amount of (*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol•$AgNO_3$ in 1 ml methanol was determined by ^{1}HNMR as 0.0211 mmol. At the meantime, another 1ml of this methanol solution was dried under reduced pressure. This neat sample of (*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol•$AgNO_3$ was heated at 30°C in an oil bath over 3 days. After that, this sample was mixed with 2.40 mg 1,3,5-trimethoxybenzene and ^{1}HNMR was taken in CD_3CN. The amount of (*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol•$AgNO_3$ was determined as 0.0204 mmol. Overall, there was 97% fidelity for (*E*)-(3,4,7,8-tetrahydro-2H-oxocin-2-yl)methanol•$AgNO_3$ after 3 days.

Stability studies for *trans*-cyclooctene silver complexes = in solution.

Stability test of ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol•$AgNO_3$ (s-TCO•$AgNO_3$) in methanol. ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol•$AgNO_3$ (4.00 mg) was mixed with 1,3,5-trimethoxybenzene (4.00 mg). The mixture was dissolved in 0.8 ml CD_3OD and ^{1}HNMR was taken as time = 0. The sample was then heated at 30°C in an oil bath over 3 days before another ^{1}HNMR spectrum was taken (as time = 3 days). Overall there was 98% fidelity for ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol•$AgNO_3$ (s-TCO•$AgNO_3$) after 3 days.

Stability test of ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol•$AgNO_3$ (s-TCO•$AgNO_3$) in DMSO. ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol•$AgNO_3$ (4.00 mg) was mixed with 1,3,5-trimethoxybenzene (4.00 mg). The mixture was dissolved in 0.8 ml DMSO-d_6 and ^{1}HNMR was taken as time=0 spectrum. The sample was then heated at 30°C in an oil bath over 3 days before another ^{1}HNMR spectrum was taken. Overall there was 96% fidelity for ((1R,8S,9r, *E*)-bicyclo[6.1.0]non-4-en-9-yl)methanol•$AgNO_3$ (s-TCO•$AgNO_3$) after 3 days.

Stability test of ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol •$AgNO_3$(d-TCO•$AgNO_3$) in methanol and DMSO. To ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol (15.6 mg, 0.847 mmol, 1.00 equiv) was added an acetonitrile (0.67 ml, 23.0 mg/ml) solution of silver nitrate (15.8 mg, 0.0931mmol, 1.10 equiv). The mixture was stirred at room temperature for 15 min. The mixture was concentrated under reduced pressure. The silver complex was dissolved in acetonitrile (3 ml). 1.0 ml of the acetonitrile solution was taken and concentrated under reduced pressure, mixed with 3.90 mg 1,3,5-trimethoxybenzene and dissolved in 0.8 ml CD_3OD. ^{1}HNMR spectrum was taken as time=0. The sample was heated at 30°C in an oil bath over 3 days. After that, another ^{1}HNMR spectrum was taken. Overall, there was 95% fidelity for ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol•$AgNO_3$ after 3 days.

Another 1.0 ml of the acetonitrile solution was taken and concentrated under reduced pressure, mixed with 5.20 mg 1,3,5-trimethoxybenzene and dissolved in 0.8 ml DMSO-d_6. ^{1}HNMR spectrum was taken as time=0. The sample was heated at 30 °C in an oil bath over 3 days. After that, another ^{1}HNMR spectrum was taken. Overall, there was 94% fidelity for ((2r,3aR,9aS, *E*)-3a,4,5,8,9,9a-hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol•$AgNO_3$ after 3 days.

Decomplexation experiment. In an NMR experiment, Ag-s-TCO-Halo (**2e**) (5 mg) was dissolved in D_2O (0.9 mL), and then added to this solution with aq. NaCl (0.1 mL, 100 mM). The final concentration of the Ag-s-TCO-Halo (**2e**) was 8.7 mM, and of NaCl was 10 mM. Within 3 minutes, the ^{1}H NMR was remeasured, and no signal was observed. The result is consistent with complete decomplexation of silver, as s-TCO-Halo (**1e**) is insoluble in D_2O. Upon extraction with C_6D_6, silver free s-TCO-Halo (**1e**) was recovered and observed by ^{1}H NMR.

Preparation of samples for ligand exchange studies by ^{1}H NMR. To a solution of **1f** (3.0 mg, 0.0094 mmol) in 0.8 mL MeOH was added 0.37 mL of a 37.8 mM methanolic stock solution of $AgNO_3$ (2.4 mg, 0.014 mmol). The mixture was allowed to stir for 15 min, and then concentrated under reduced pressure. The mixture was then dissolved in CD_3OD and ^{1}H NMR spectrum of a solution where the ratio of **1f**: silver nitrate was 1.00 :1.50. Similar procudures were used to produce ^{1}H NMR spectra where the ratios of **1f**: silver nitrate was 1.00 :1.00, 1.00 : 0.50 and 1.00 : 0.25

HeLa cells preparation. HeLa cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (HI FBS), 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-alanyl-L-glutamine dipeptide (glutaMAX), and 100 U/mL penicillin-streptomycin to near confluency in five T175 flasks. Media was aspirated cells washed three times with 5 mL DPBS and treated with 5 mL 0.25% trypsin-EDTA at 37 °C and 5% CO_2, until cells are dislodged from flask bottom. Trypsin was quenched with 5 mL of complete DMEM and triturated to remove clumps. Cells were centrifuged at 1200 rpm for 3 minutes, supernatant removed, and the cell pellet resuspended in 10 mL complete DMEM. Cells were counted with a Countess device, adjusted to $6x10^5$ cells/mL with complete DMEM, and 2 mL added per well of 6-well plates (for in-gel experiment) or 6 cm MatTek dishes for imaging experiments. Cells were allowed to adhere overnight at 37 °C and 5% CO_2.

HeLa cells transfection. Media was aspirated and cells were washed three times with 1 mL DPBS and 1 mL antibiotic-free complete DMEM was added to each well or MatTek dish. Opti-MEM/lipofectamine 2000 solution was prepared by mixing together 62.5 µL Opti-MEM and 1.5 µL lipofectamine 2000 per well to be transfected and the mixture incubated for 5 minutes at room temperature. Opti-MEM and Halo-H2B-GFP plasmid DNA were prepared by mixing 62.5 µL Opti-MEM and 0.5 µg DNA per well to be transfected and the mixture was incubated for 5 minutes at room temperature. The lipofectamine 2000 and DNA mixtures were then combined and incubated for 20 minutes at room temperature. 125 µL of the lipofectamine 2000/DNA mixture was added to each well and after briefly rotating the plates to ensure proper mixing in the wells, the cells were incubated at 37 °C and 5% CO_2 for 3 hours. Media was then aspirated from each well and replaced with 2 mL complete DMEM and the cells incubated overnight at 37 °C and 5% CO_2.

HaloTag conjugation. Media was aspirated from transfected cells, which were then washed three times with 1 mL DPBS and 1 mL complete DMEM containing 2 µM MeTz-Halo was added to each well. For a negative control, 1 mL complete DMEM containing DMSO control was added to three wells. For a positive control, 1 mL complete DMEM containing 1 µM TAMRA-Halo was added to three wells. Cells were incubated at 37 °C and 5% CO_2 for 30 minutes, media aspirated, and cells washed three times with 1 mL DPBS.

In-gel fluorescence analysis of time course tetrazine ligation. DPBS was aspirated from each well and replaced with 1 mL complete DMEM containing 2 uM s-TCO-TAMRA•$AgNO_3$ and cells were incubated at 37 °C and 5% CO_2 for time periods varying from 10 seconds to 2 hours. At the end of indicated time, reactions were quenched by addition of TCO-NH_2 (1 mL, 100 uM) . Media was aspirated from wells, TCO-NH_2 (1 mL, 100 uM) added to each well, and cells were scraped and transferred to 1.5 mL Eppendorf tubes. Cells were pelleted by centrifugation at 5000xg for 3 minutes and supernatant aspirated.

Protein gel electrophoresis, in-gel fluorescence, and western blotting. Cell pellets were lysed in 50 µL 1% SDS in DPBS with several pulses of a probe sonicator and quantitated with a Pierce BCA assay. 30 µg aliquots of each sample were adjusted to a total volume of 39 µL with 1% SDS and 15 µL 4x LDS and 6 µL NuPAGE 10x sample reducing agent were added. Samples were then heated at 72 °C for 10 minutes. 18 µL of each sample was then loaded per lane of 12 well NuPAGE 4-12% Bis-Tris gel ran in NuPAGE MES running buffer at 140 V for 60 minutes. Gels were then removed from cassettes, transferred to distilled water, and immediately scanned on Typhoon FLA9500 variable mode imager with a long-pass LPG filter. Proteins were then transferred to nitrocellulose membrane using iBlot2 gel transfer system and membranes incubated in 10 mL Pierce Protein-Free (PBS) blocking buffer for 1 hour at room temperature. Blocking buffer was removed and additional 5 mL blocking buffer containing 1:1,000 rabbit anti-HaloTag polyclonal antibody was added and blot incubated at room temperature for 1 hour. The membrane was washed with 15 mL TBS containing 0.1% Tween 20 (TBST) for 10 minutes on rotating platform three times. 10 mL TBST containing 1:10,000 goat anti-rabbit IRDye 800CW secondary antibody was added and allowed to incubate for 1 hour at room temperature. The membrane was washed with 15 mL TBST for 10 minutes on rotating platform five times. The membrane was scanned on Licor Odyssey CLx system. ImageQuant TL 1D v8.1 software was used to determine intensity of in-gel fluorescence bands and normalized to total HaloTag protein as a % of positive control.

Confocal microscopy. DPBS was aspirated from well and replaced with 1 mL of complete DMEM containing 500 nM s-TCO-TAMRA and cells incubated for 60 min at 37 °C and 5% CO_2. TCO-NH_2 (1 mL, 100 uM) was then added to each well, and cells washed twice with TCO-NH_2 (1 mL, 100 uM). Cells were incubated in 1 mL of fresh phenol red-free complete DMEM for an hour, and media changed after 30 minutes with fresh phenol red-free complete DMEM before imaging on Zeiss Axio Observer Z1 inverted microscope with a Yokogawa CSU-X1M 5000 dual camera spinning disk system and acquired with a Photometrics Evolve 512 Delta camera using Zen Blue 2012 software. Cells were visualized using a 40x/1.3 numerical aperture (NA) objective and illuminated with the 488 and 561 laser lines. Green and red emission was collected sequentially (BP 525/50 and BP 617/73 respectively).

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Graphical Abstract