Journal of Visualized Experiments

Design, synthesis, and photochemical properties of clickable caged compounds

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60021R1
Full Title:	Design, synthesis, and photochemical properties of clickable caged compounds
Keywords:	caged compound; Synthesis; photochemistry; click chemistry; prodrug; Chemical Biology; chemical probe
Corresponding Author:	Toshiaki Furuta Toho University Funabashi, Chiba JAPAN
Corresponding Author's Institution:	Toho University
Corresponding Author E-Mail:	furuta@biomol.sci.toho-u.ac.jp
Order of Authors:	Akinobu Z. Suzuki
	Yukiko Shiraishi
	Hanami Aoki
	Hirona Sasaki
	Rei Watahiki
	Toshiaki Furuta
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Funabashi city, Chiba, Japan

TITLE:

Design, Synthesis, and Photochemical Properties of Clickable Caged Compounds

AUTHORS AND AFFILIATIONS:

Akinobu Z. Suzuki, Yukiko Shiraishi, Hanami Aoki, Hirona Sasaki, Rei Watahiki, and Toshiaki Furuta

Department of Biomolecular Science, Faculty of Science, Toho University, Funabashi, Chiba, Japan

Email addresses of co-authors:

Akinobu Z. Suzuki (akinobu@biomol.sci.toho-u.ac.jp)
Yukiko Shiraishi (050238ft@biomol.sci.toho-u.ac.jp)
Hanami Aoki (6318001a@st.toho-u.ac.jp)
Hirona Sasaki (6319007s@st.toho-u.ac.jp)
Rei Watahiki (6319017w@st.toho-u.ac.jp)

Corresponding author:

Toshiaki Furuta (furuta@biomol.sci.toho-u.ac.jp)

KEYWORDS:

caged compounds, synthesis, photochemistry, click chemistry, prodrug, chemical biology, chemical probes

SUMMARY:

A protocol for the synthesis and measurement of the photochemical properties of modular caged compounds with clickable moieties is presented.

ABSTRACT:

Caged compounds enable the photo-mediated manipulation of the cell physiology with high spatiotemporal resolution. However, the limited structural diversity of currently available caging groups and the difficulties in synthetic modification without sacrificing their photolysis efficiencies are obstacles to expanding the repertoire of caged compounds for live cell applications. As the chemical modification of coumarin-type photo-caging groups is a promising approach for the preparation of caged compounds with diverse physical and chemical properties, we report a method for the synthesis of clickable caged compounds that can be modified easily with various functional units via the copper(I)-catalyzed Huisgen cyclization. The modular platform molecule contains a (6-bromo-7-hydroxycoumarin-4-yl)methyl (Bhc) group as a photo-caging group, which exhibits a high photolysis efficiency compared to those of the conventional 2-nitrobenzyls. General procedures for the preparation of clickable caged compounds containing amines, alcohols, and carboxylates are presented. Additional properties such as the water solubility and cell targeting ability can be readily incorporated into clickable caged compounds. Furthermore, the physical and photochemical properties, including the photolysis quantum yield, were measured and were found to be superior to those of the corresponding Bhc caged compounds. The described protocol could therefore be considered a potential solution for the lack of structural diversity in the available caged compounds.

INTRODUCTION:

Caged compounds are designed synthetic molecules whose original functions are temporally masked by covalently attached photo-removable protecting groups. Interestingly, caged compounds of biologically relevant molecules provide an indispensable method for the spatiotemporal control of the cellular physiology[1–6]. In 1977, Engels and Schlaeger reported the 2-nitrobenzyl ester of cAMP as a membrane permeable and photolabile derivative of cAMP[7]. The following year, Kaplan reported the 1-(2-nitrophenyl)ethyl ester of ATP (NPE-ATP) and named this compound "caged" ATP[8]. Since then, a range of photochemically removable protecting groups such as 2-nitrobenzyls, *p*-hydroxyphenacyls[9], 2-(2-nitrophenyl)ethyls[10,11], 7-nitroindolin-1-yls[12,13], and (coumarin-4-yl)methyls[14–16] have been used for the preparation of caged compounds.

The synthesis of caged compounds with desirable additional properties such as membrane permeability, water solubility, and cellular targeting ability would be expected to facilitate cell biological applications. Since the physical and photochemical properties of these molecules depend primarily on the chemical structure of the photochemically removable protecting groups used to prepare them, a diverse repertoire of photo-caging groups is required. However, the structural diversity of currently available caging groups that exhibit high photolysis efficiencies is limited. This could be an obstacle to increasing the use of caged compounds.

To address this issue, the repertoire of photo-caging groups has been expanded by the chemical modification of existing photoremovable protecting groups or the design of new photolabile chromophores with superior photophysical and photochemical properties. Examples include nitrodibenzofuran (NDBF)[17], [3-(4,5-dimethoxy-2-nitrophenyl)-2-butyl] (DMNPB)[18,19], a calcium-sensitive 2-nitrobenzyl photocage[20], substituted coumarinylmethyls (DEAC450[21], DEAdcCM[22], 7-azetidinyl-4-methylcoumarin[23], and styryl coumarins[24]), cyanine derivatives (CyEt-pan)[25], and BODIPY derivatives[26,27].

In addition, we previously developed the (6-bromo-7-hydroxycoumarin-4-yl)methyl (Bhc) group and successfully synthesized various caged compounds of neurotransmitters[28], second messengers[29,30], and oligonucleotides[31–33] exhibiting large one- and two-photon excitation cross-sections. If additional properties can be installed easily into the caging groups without compromising their photosensitivity, then the repertoire of caged compounds can be expanded[34–39]. We therefore designed modular caged compounds that comprise three parts, namely the Bhc group as a photo-responsive core, chemical handles for the installation of additional functionalities, and the molecules that are to be masked[40,41].

Thus, this article provides a practical method for the preparation of caged compounds of biologically relevant molecules. The present protocol describes methods for the preparation of a clickable platform for photo-caging groups, the introduction of additional functionalities to expand the repertoire of caged compounds, the measurement of their physical and photochemical properties, and the cell-type selective targeting of a clickable caged compound for further cellular application.

PROTOCOL:

1. Synthesis of the modular caging paBhc group for clickable caged compounds[28,41]

1.1. Preparation of (6-bromo-7-hydroxycoumarin-4-yl)methyl chloride (Bhc-CH_2Cl)

1.1.1. Place 4-bromoresorcinol (9.742 g, 51.5 mmol) in a 100 mL round-bottomed flask equipped with a stirrer bar.

1.1.2. Add conc. H_2SO_4 (98%, 30 mL) to the flask and stir the mixture to dissolve.

1.1.3. Add ethyl 4-chloroacetoacetate (10 mL, 74 mmol) dropwise.

1.1.4. Continue stirring the mixture at ambient temperature for 5 days.

1.1.5. Separately, place crushed ice cubes (~200 mL) in a 500 mL Erlenmeyer flask.

1.1.6. Pour the reaction mixture into the ice and stir vigorously for 30 min until a finely powdered precipitate is obtained.

1.1.7. Collect the precipitate via vacuum filtration. Wash the light brown precipitate with water five times.

1.1.8. Dry the precipitate under vacuum overnight to yield BhcCH_2Cl as a light brown powder (13.57 g, 46.9 mmol).

1.2. Preparation of (6-bromo-7-hydroxycoumarin-4-yl)methanol (BhcCH_2OH)

1.2.1. Place the prepared BhcCH_2Cl (1.1440 g, 3.95 mmol) and 1 M HCl (300 mL) in a 1 L round-bottomed flask equipped with a Dimroth condenser. Stir the mixture at 140 °C for 5 days. After this time, cool the mixture to ambient temperature.

1.2.2. Remove the water from the reaction by rotary evaporation under vacuum to yield BhcCH_2OH (**1**) as a light brown powder (1.0359 g, 3.82 mmol, 97% yield).

NOTE: The use of 250 mL of 1 M HCl per 1 g of BhcCH_2Cl gives a satisfactory result.

1.3. Preparation of paBhcCH_2OH (2) via the Mannich reaction

1.3.1. Place paraformaldehyde (446.4 mg, 14.9 mmol) in a 50 mL round-bottomed flask. Add anhydrous ethanol (5 mL) and *N*-methylpropargylamine (1.25 mL, 14.8 mmol) to the flask.

1.3.2. Stir the mixture at ambient temperature for 1 h under an Ar atmosphere.

1.3.3. Add BhcCH_2OH (**1**) (1.367 g, 5.04 mmol) to the flask. Heat the mixture to 80 °C with a block

heater apparatus, and continue stirring the mixture at 80 °C for 2 h under an Ar atmosphere.

1.3.4. Stop the block heater and cool the reaction mixture to room temperature.

1.3.5. Collect the resulting light brown-yellow precipitate by vacuum filtration. Wash the precipitate twice with a small amount of anhydrous ethanol (1 mL each time).

1.3.6. Remove the excess ethanol under vacuum to yield paBhcCH_2OH (**2**) (1.393 g, 3.96 mmol).

2. Preparation of clickable caged compounds

NOTE: The following procedures can be applied to the preparation of other clickable caged compounds containing hydroxyl, amino, and carboxylate functional groups.

2.1. General Procedure 1: Preparation of a clickable caged amine

2.1.1. Place paBhcCH_2OH (**2**) (709.6 mg, 2.02 mmol) and *N*,*N'*-carbonyl diimidazole (CDI, 397.6 mg, 2.45 mmol) in a 30 mL round-bottomed flask. Add dry CH_2Cl_2 (6 mL) and stir the solution at ambient temperature for 1 h.

2.1.2. Add 4-dimethylaminopyridine (4-DMAP, 324.8 mg, 2.66 mmol) and *tert*-butyl (6-aminohexyl)carbamate (533.1 mg, 2.46 mmol). Stir the solution at ambient temperature for 3 h.

2.1.3. Remove the solvent and other volatile materials using a rotary evaporator under vacuum. Purify the residue directly using silica gel flash column chromatography.

2.2. General Procedure 2: Preparation of a clickable caged alcohol

2.2.1. Place paclitaxel (PTX, 48.7 mg, 0.057 mmol) in a 30 mL round-bottomed flask equipped with a three-way stopcock and an Ar balloon. Add dry CH_2Cl_2 (1 mL), 4-DMAP (17.1 mg, 0.14 mmol), and 4-nitrophenyl chloroformate (26.0 mg, 0.13 mmol).

2.2.2. Stir the solution at ambient temperature for 2.5 h under an Ar atmosphere.

2.2.3. Add 4-DMAP (15.7 mg, 0.13 mmol) and paBhcCH_2OH (**2**) (39.1 mg, 0.111 mmol) to the solution. Continue stirring the mixture at ambient temperature for 17 h.

2.2.4. Add $CHCl_3$ (10 mL) and 15% aqueous $NaHCO_3$ (5 mL) to the mixture. Stir the mixture vigorously for approximately 3 min. Remove the aqueous layer with a pipette.

2.2.5. Add 0.5 M citric acid (5 mL) to the flask containing the organic layer. Stir the mixture and remove the aqueous layer as above.

2.2.6. Separate the organic layer using a phase separation column. Remove the solvents with a

rotary evaporator under vacuum. Purify the product using standard silica gel flash column chromatography.

2.3. General Procedure 3: Preparation of a clickable caged carboxylic acid

2.3.1. Dissolve arachidonic acid (33.0 µL, 0.100 mmol), paBhcCH_2OH (**2**) (39.6 mg, 0.112 mmol), and 4-DMAP (14.1 mg, 0.115 mmol) in dry CH_2Cl_2 (2 mL). Add *N*,*N*′-diisopropylcarbodiimide (DIPC, 17.0 µL, 0.110 mmol) and stir the solution at ambient temperature for 140 min.

2.3.2. Remove the solvent under vacuum. Purify the residue directly using silica gel flash column chromatography.

3. Installation of a functional unit into the clickable caged compounds

3.1. Dissolve copper(II) sulfate pentahydrate (249 mg) in ion-exchanged water (IEW, 10 mL) to give a 0.1 M $CuSO_4$ solution.

3.2. Dissolve 2′-paBhcmoc-PTX (8.0 mg, 6.5 µmol), tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, 17.5 mg, 40.3 µmol), sodium L-ascorbate (162.4 mg, 0.825 mmol), and 15-chloro-3,6,9-trioxapentadecyl azide (3.1 mg, 11 µmol) in a mixed solvent of 0.1 M phosphate buffer (2.5 mL, pH 7.2) and dimethyl sulfoxide (DMSO, 0.5 mL).

3.3. Add the 0.1 M $CuSO_4$ solution (81.2 µL, 8.1 µmol) to the reaction mixture. Stir the mixture at ambient temperature for 80 min. Monitor the progress of the reaction using high-performance liquid chromatography (HPLC).

3.4. Dissolve the precipitates by adding a 75% acetonitrile/water solution (3.5 mL). Apply the resulting solution directly to the semi-preparative HPLC system to purify the desired product.

NOTE: Solubilization of the reaction mixture by the addition of *tert*-butanol can accelerate the progression of the reaction.

4. Photolytic uncaging reaction of the caged compounds

4.1. Preparation of the stock solutions

4.1.1. Dissolve the desired caged compound (5 µmol) in DMSO (500 µL) to prepare a 10 mM stock solution. Dispense an aliquot of each solution (10 µL) into a 1.5 mL microcentrifuge tube and store in a freezer (−20 °C) until just before use.

4.1.2. 6 mM $K_3[Fe(C_2O_4)_3]$ (100 mL): Dissolve recrystallized potassium ferrioxalate (0.295 g, 0.675 mmol) in 80 mL of water. Add 0.5 M H_2SO_4 (10 mL) and an appropriate amount of IEW to make up the volume to 100 mL.

NOTE: Potassium ferrioxalate should be purified via recrystallization from hot water and stored in the dark. Recrystallized potassium ferrioxalate is obtained as the trihydrate; therefore, its formula is $K_3[Fe(C_2O_4)_3]\cdot 3H_2O$ and a formula weight of 491.24 should be considered during preparation of the stock solution. Check the purity of the 6 mM solution by measuring its absorption at 510 nm. If the absorbance is <0.02, it is suitable for use in the experiment.

4.1.3. 0.1% Buffer-phen (30 mL): Dissolve $NaOAc\cdot 3H_2O$ (7.35 g), 1,10-phenanthroline (phen)·H_2O (30 mg), and conc. H_2SO_4 (0.9 mL) in IEW (20 mL). Add IEW to make up the volume to 30 mL.

NOTE: The solution contains 1.8 M NaOAc, 0.54 M H_2SO_4, and 0.1% 1,10-phenanthroline.

4.2. Measurement of the number of photons using ferrioxalate actinometry

4.2.1. Place 6 mM $K_3[Fe(C_2O_4)_3]$ (V_1 L) in a quartz cuvette. Irradiate the solution with 350 nm light for 5 s.

4.2.2. Transfer the irradiated solution to a Pyrex cuvette with an l [cm] path length.

4.2.3. Add 0.1% Buffer-phen (V_2 L) to the irradiated sample solution and mix well by pipetting. Measure the absorbance of the sample at 510 nm. Calculate the average absorption change per unit time (ΔA_{510} [s^{-1}]).

4.2.4. Calculate the number of moles of generated Fe^{2+} ions per unit time according to the following equation:

$$nFe^{2+}\ [mol\ s^{-1}] = ((V_1 + V_2)\ [L] \times \Delta A_{510}\ [s^{-1}])/(l\ [cm] \times \varepsilon_{510}\ [L\ mol^{-1}\ cm^{-1}]),$$

where ($V_1 + V_2$) is the volume of the sample for the absorption measurement, l is the optical path length of the cuvette, and ε_{510} is the molar absorptivity of the Fe^{2+}-phen complex at 510 nm.

NOTE: In the typical experimental conditions, values of $V_1 = 2.0 \times 10^{-3}$ L, $V_2 = 0.33 \times 10^{-3}$ L, $l = 1.0$ cm, and $\varepsilon_{510} = 1.1 \times 10^4$ L mol^{-1} cm^{-1} were used.

4.2.5. Calculate the number of moles of photons that reach the sample (I_0) using the following formula:

$$I_0\ [einstein\ cm^{-2}\ s^{-1}] = nFe^{2+}/\Phi_{350}$$

where Φ_{350} is the quantum efficiency of photoreduction of the ferrioxalate at 350 nm.

NOTE: Although the quantum efficiency of the potassium ferrioxalate actinometer at 350 nm is not reported, the reported value of 1.25[42] at 358 nm was employed.

4.3. Quantum efficiency measurements at 350 nm

4.3.1. Dilute the sample stock solution (in DMSO, 10 µL) with K-MOPS buffer (pH 7.2, 10 mL) to give a 10 µM solution in K-MOPS containing 0.1% DMSO.

NOTE: K-MOPS buffer consisted of 100 mM KCl and 10 mM 3-(*N*-morpholino)propanesulfonic acid (Mops) titrated to pH 7.2 with KOH.

4.3.2. Transfer an aliquot of the solution (V_1 L) into the same cuvette used in the photoreaction of the chemical actinometer. Irradiate the sample solution using the same setup as described in step 4.2.1.

4.3.3. Remove an aliquot (50 µL) from the irradiated solution periodically and analyze using HPLC.

4.3.4. Determine the irradiation time, in seconds, in which 90% of the starting material reacted ($t_{90\%}$) by fitting plots of the time-dependent disappearance of the starting material.

NOTE: The absorbance of the irradiated sample must be maintained at <0.1 so that the inner filtering of the radiation can be neglected. It is desirable that the photolytic consumption of the starting material can be approximated by single-exponential decay so that there is no undesired secondary effect that interferes with the photolysis process.

4.3.5. Calculate the quantum yield of disappearance (Φ_{dis}) using the following equation[28]:

$$\Phi_{dis} = 1/(t_{90\%} \times I_0 \times \sigma_{350})$$

where $t_{90\%}$ [s] is the irradiation time in which 90% of the starting material was consumed, I_0 [einstein cm^{-2} s^{-1}] is the number of moles of photons, and σ_{350} [cm^2 mol^{-1}] is the decadic extinction coefficient of the sample at 350 nm.

NOTE: σ_{350} [cm^2 mol^{-1}] = 10^3 ε_{350} [M^{-1} cm^{-1}].

5. Targeting of a clickable caged compound with a HaloTag ligand

NOTE: Prior to use, maintain the HeLa cells in Dulbecco's modified Eagle medium (DMEM, low glucose, sodium pyruvate, L-glutamine) supplemented with 10% fetal bovine serum (FBS) containing 1% antibiotics (streptomycin sulfate, penicillin G, and amphotericin) at 37 °C and 5% CO_2.

5.1. Remove the medium and trypsinize the cells by treating with trypsin-ethylenediaminetetraacetic acid (EDTA, 1 mL) at 37 °C for 1 min. Add DMEM (4 mL) to the cells and re-suspend the cells by pipetting gently. Seed approximately 5×10^5 cells per dish into 35 mm glass bottom dishes in DMEM (2 mL) 24 h before transfection.

5.2. For four dishes, in a 1.5 mL microcentrifuge tube, dilute the plasmid DNA (pcDNA3-Halo-

EGFR, 14 µg) in the reduced serum medium (700 µL). Separately, dilute the lipofection reagent (5 µL) in the reduced serum medium (150 µL) into each of four tubes and allow them stand at ambient temperature for 5 min.

5.3. Add a portion of the diluted plasmid DNA (150 µL) to each of the diluted lipofection reagent samples. Incubate at ambient temperature for 5 min.

5.4. After maintaining the cells at 37 °C and 5% CO_2 for 24 h, aspirate the DMEM and rinse the cells with phosphate-buffered saline (PBS, 2 mL). Add the reduced serum medium (1.5 mL).

5.5. Add the plasmid-lipofection reagent (150 µL) complex to each dish. Maintain the cells at 37 °C and 5% CO_2 for 48 h.

5.6. Aspirate the medium, add a portion of freshly prepared DMEM (1 mL) containing 2 µM paBhc-hex-FITC/Halo, and incubate the cells at 37 °C and 5% CO_2 for 30 min.

5.7. Aspirate the medium containing the caged compound and rinse the cells twice with PBS+ (1 mL per rinse) to remove any unbound compounds. Add the reduced serum medium (500 µL) and incubate the cells at 37 °C and 5% CO_2 for 30 min to remove the compounds that entered the cells.

NOTE: PBS+ is phosphate-buffered saline supplemented with 2 mM $CaCl_2$ and 1 mM $MgCl_2$.

5.8. Remove the medium and rinse the cells twice with PBS+ (1 mL). Add a portion of a medium (1 mL) that does not contain phenol red. Record fluorescence images by laser scanning confocal fluorescence microscopy.

6. Photomediated modulation of a kinase localization using a clickable caged compound

NOTE: Prior to use, maintain the CHO-K1 cells in Ham's F-12 medium supplemented with 10% FBS at 37 °C and 5% CO_2.

6.1. Prepare a 100× working solution (1 mM)of paBhc-AA (**5**) in DMSO.

NOTE: A 10 mM stock solution of the compound is prepared and stored in a freezer (-20 °C).

6.2. Seed approximately 5 × 10^5 cells per dish into 35 mm glass bottom dishes in DMEM (2 mL) 24 h before transfection.

6.3. Transfect CHO-K1 cells with a plasmid coding for GFP-DGKγ 48 h before the uncaging experiments.

NOTE: Transfection is performed according to steps 5.2–5.5.

6.4. Replace the medium with a reduced serum medium (2 mL). Add the 100× paBhc-AA working solution (20 µL) and incubate the cells at 37 °C and 5% CO_2 for between 5 min and 1 h.

NOTE: The loading time depends on the compound employed.

6.5. Place the cells on the objective stage of an inverted fluorescent microscope equipped with a dual light source fluorescence illuminator.

6.6. Take a fluorescent image every 10 s. Irradiate the cells with 330–385 nm light through a microscope objective for an appropriate time. Alternatively, irradiate the cells with 405 nm light using a Xe lamp through flexible quartz fibers.

6.7. Continue to record fluorescent images for 10 min.

REPRESENTATIVE RESULTS:

Clickable caged compounds of some biologically interesting molecules, including arachidonic acid and paclitaxel, were successfully synthesized (**Figure 1**)[28,41]. Additional properties such as the water solubility and cellular targeting ability were introduced into paBhcmoc-PTX via the copper(I)-catalyzed Huisgen cyclization ("Click" reaction) (**Figure 2**). These clickable caged PTXs were then photolyzed to produce their parent PTXs upon irradiation at 350 nm (**Figure 3**), and the physical and photochemical properties of the clickable caged compounds are summarized in Table 1. The quantum yields of clickable caged compounds 2'-glc-paBhcmoc-PTX (Φ_{dis} 0.14) and paBhc-AA (Φ_{dis} 0.083) were more than twice those of conventional Bhc caged compounds 2'-Bhcmoc-PTX (Φ_{dis} 0.040) and Bhc-AA (Φ_{dis} 0.038)[43]. In addition, an improved water solubility was observed for 2'-glc-paBhcmoc-PTX, which contains a glucose moiety.

In live cell experiments, the targeting of paBhc-hex-FITC/Halo to the cultured mammalian cells transiently expressing a fusion protein of a HaloTag protein and epidermal growth factor receptor (EGFR) was achieved successfully. Green fluorescence of the fluorescein moiety of paBhc-hex-FITC/Halo was observed on the cell membrane (**Figure 4**). Photo-mediated modulation of the subcellular localization of a kinase was achieved using a paBhc caged compound. The translocation of diacylglycerol kinase γ (DGKγ) has been reported to be activated in the presence of arachidonic acid (AA)[44]. CHO-K1 cells transiently expressing GFP-DGKγ were treated with either AA or paBhc-AA (**5**). Addition of AA caused the modulation of the subcellular localization of DGKγ (**Figure 5A,B**). Similar changes in the localization of DGKγ were observed for the paBhc-AA-treated cells after exposure to UV light (**Figure 5C,D**).

FIGURE AND TABLE LEGENDS:

Figure 1: Preparation of the clickable caged compounds. (**A**) Reagents and conditions: a. ethyl 4-chloroacetoacetate/conc. H_2SO_4/rt/7 days/91% yield, b. 1 M HCl/reflux/3 days/97% yield. c. *N*-methylpropargylamine /HCHO/EtOH, then add (**1**) and heat at reflux for 17 h/79% yield. (**B**) Syntheses of the clickable caged amine, PTX, and arachidonic acid.

Figure 2: Installation of functional units into clickable caged compounds. (**A**) Synthesis of a

water-soluble caged PTX via the copper(I)-catalyzed Huisgen cyclization. (**B**) Structures of clickable caged compounds containing the HaloTag ligand for cellular targeting.

Figure 3: Photolysis of 2'-glc-paBhcmoc-PTX (6). Samples (10 μM) in K-MOPS solution (pH 7.2) were irradiated at 350 nm. (**A**) Typical HPLC traces for the photolysis of **6** (measured at 254 nm). Samples were analyzed at the specified irradiation time. (**B**) Time course for the photolysis of **6**. Blue circles show the consumption of **6**. The solid line shows the least-squares curve fit for a simple decaying exponential for **6**. Red squares show the yield of PTX. The error bars represent the standard deviation (±SD).

Figure 4: Fluorescence images of cultured mammalian cells incubated with paBhc-hex-FITC/Halo (8). Cells transfected with pcDNA3-Halo-EGFR were incubated with a 2 μM solution of compound **8** at 37 °C for 30 min. The images were obtained after repeated washing with PBS+. Mock-treated HEK293T cells (**A**: differential interference contrast (DIC) image and **D**: fluorescence image). HEK293T cells (**B** and **E**) and HeLa cells (**C** and **F**) transiently expressing Halo-EGFR (**B** and **C**: DIC images and **E** and **F**: fluorescence images).

Figure 5: Fluorescence images after UV irradiation of the CHO-K1 cells incubated with Bhc caged arachidonic acid. CHO-K1 cells were transfected with a fusion protein DGKγ-EGFP. (**A**) A fluorescence image of the transfected cells. (**B**) 100 s after the addition of a 10 μM solution of arachidonic acid. (**C**) Cells were incubated with a 10 μM solution of paBhc-AA (**5**) at 37 °C for 5 min. (**D**) 100 s after 20-s UV irradiation (330–385 nm).

Table 1: Physical and photochemical properties of the clickable caged compounds.
a. Absorption maximum (nm), b. Molar absorptivity at λ_{max} (M^{-1} cm^{-1}), c. Quantum yield of the disappearance of the starting materials at 350 nm, d. The product of molar absorptivity and the quantum yield of disappearance at 350 nm, e. The concentration of the saturated solution in K-MOPS (pH 7.2) (μg mL^{-1}).

DISCUSSION:

We previously developed Bhc caged compounds of various biologically active molecules that exhibit high photolytic efficiencies[28,45–47]. With the aim of expanding the repertoire of Bhc caging groups, we also reported platforms of modular caged compounds that can be modified easily by the introduction of various functional units[32,40,41]. The present protocol therefore represents a method for the synthesis of a clickable precursor of Bhc caging groups that can be modified via the copper(I)-catalyzed Huisgen cyclization. The synthesis of the clickable precursor, paBhcCH_2OH (**2**), was achieved via a four-step reaction sequence starting from the commercially available 4-bromoresorcinol (**Figure 1A**). The advantage of the present protocol is that no laborious purification steps (e.g., column chromatographic separations) are required.

As clickable precursor paBhcCH_2OH (**2**) can be used to mask various functional groups, clickable caged compounds of amines, alcohols, and carboxylic acids were synthesized using **2** as the precursor (**Figure 1B**). Amines were modified as their carbamates while alcohols were modified as their carbonates. In general procedures 1 and 2, CDI was used for the preparation of clickable

carbamates, while 4-nitrophenyl chloroformate was used for the preparation of carbonates. As indicated by the reaction mechanism, both reagents can be used for the preparation of carbamates and carbonates. It should also be noted that the yield of the desired caged compound depends on the chemical structure of the molecule to be caged. Other examples can be seen in our previous reports[28,30,33,48].

Click modification was then performed using a slight modification of the reported procedure[49]. The addition of tris(triazolylmethyl)amine-based ligands is necessary to obtain the desired products in good to high yields. Since a variety of azides are readily available both from commercial sources and from literature procedures, we can prepare various modular caged compounds with additional properties such as water solubility and cellular targeting ability (**Figure 2**).

The quantum yield of photolysis was then measured according to a reported procedure[28,50]. **Figure 3** shows that the photolytic consumption of 2′-glc-paBhcmoc-PTX and the release of PTX were approximated by single-exponential decay and rise, respectively, suggesting no inner filtering of the radiation or undesired secondary effects. Improved photolysis quantum yields (Φ) and photolysis efficiencies ($\varepsilon\Phi$) were observed for the clickable paBhc caged compounds compared to those of the previously reported Bhc caged compounds (**Table 1**)[41,43]. Since the photolysis efficiencies ($\varepsilon\Phi$) of Bhc caged compounds are more than one hundred times higher than those of 2-nitrobenzyl-type caged compounds[48], the marked improvement due to the presence of paBhc caging groups is clearly an advantage for this system.

As a proof-of-concept experiment, a hydrophilic moiety was introduced into 2′-paBhcmoc-PTX (**4**) and a cellular targeting ligand was introduced into compound **3** (**Figure 2**). The water solubility of 2′-glc-paBhcmoc-PTX was 650 times higher than that of the parent PTX (**Table 1**). Selective cellular targeting was achieved using a tag-probe system, and paBhcmoc-hex-FITC/Halo (**8**) bearing the HaloTag ligand was successfully targeted to the cell membrane of cultured mammalian cells expressing the HaloTag/EGFR fusion protein (**Figure 4**). Photo-mediated modulation of the subcellular localization of a kinase was also achieved using a clickable caged compound **5** (**Figure 5**).

In conclusion, we successfully demonstrated a method for the preparation of clickable platforms for photo-caged compounds of biologically interesting molecules that can be modified easily with additional properties, such as water solubility and a cellular targeting ability. Since the paBhc caging group can be used to prepare any molecules with modifiable functional groups, the application of the present protocol is not limited to the molecules described herein. Using a modular platform, namely the paBhc caging group, the desired caged compounds can be easily prepared, and their physical and chemical properties can be modulated via click modification.

ACKNOWLEDGMENTS:
This work was supported by JSPS KAKENHI grant number JP16H01282 (TF), a Grant-in-Aid for Scientific Research on Innovative Areas “Memory Dynamism,” and JP16H06280 (TF), Platforms for Advanced Technologies and Research Resources “Advanced Bioimaging Support.”

DISCLOSURES:

We have nothing to disclose.

REFERENCES:

1 Mayer, G., Heckel, A., Biologically active molecules with a "light switch", *Angewandte Chemistry International Edition*, **45** (30), 4900-4921 (2005)

2 Bort, G., Gallavardin, T., Ogden, D., Dalko, P. I., From One-Photon to Two-Photon Probes: "Caged" Compounds, Actuators, and Photoswitches. *Angewandte Chemistry International Edition* **52** (17), 4526-4537 (2013).

3 Klan, P., et al., Photoremovable Protecting Groups in Chemistry and Biology: Reaction Mechanisms and Efficacy. *Chemical Reviews* **113** (1), 119-191 (2013).

4 Abe, M., et al., Design and Synthesis of Two-Photon Responsive Chromophores for Near-Infrared Light-Induced Uncaging Reactions. *Synthesis-Stuttgart***, 49** (15), 3337-3346 (2017**)**.

5 Ankenbruck, N., Courtney, T., Naro, Y., Deiters, A., Optochemical Control of Biological Processes in Cells and Animals, *Angewandte Chemistry International Edition*, **57** (11), 2768 (2018)

6 Hou, Y., Zhou, Z., Huang, K., Yang, H., Han, G., Long Wavelength Light Activated Prodrug Conjugates for Biomedical Applications, *ChemPhotoChem*, 2, 1005 (2018)

7 Engels, J., Schlaeger, E. J., Synthesis, structure, and reactivity of adenosine cyclic 3',5'-phosphate benzyl triesters, Rapid photolytic release of adenosine 5'-triphosphate from a protected analogue: utilization by the Na:K pump of human red blood cell, *Journal of Medicinal Chemistry*, 1977, **20** (7), 907-911.

8 Kaplan, J. H., Forbush III, B., Hoffman, J. F., Rapid photolytic release of adenosine 5'-triphosphate from a protected analogue: utilization by the Na:K pump of human red blood cell ghosts *Biochemistry*, 1978, **17** (10), 1929-1935.

9 Park, C.-H., Givens, R. S., New Photoactivated Protecting Groups. 6. p-Hydroxyphenacyl: A Phototrigger for Chemical and Biochemical Probes, *Journal of the American Chemical Society*, **119** (10), 2453-2463 (1997).

10 Hasan, A., et al., Photolabile protecting groups for nucleosides: synthesis and photo-deprotection rates, *Tetrahedron*, **53** (12), 4247-4264 (1997).

11 Heckel, A., Mayer, G., Light regulation of aptamer activity: an anti-thrombin aptamer with caged thymidine nucleobases, *Journal of the American Chemical Society*, **127** (3), 822-823 (2005).

12 Papageorgiou, G., Corrie, J. E. T., Effects of aromatic substituents on the photocleavage of 1-acyl-7-nitroindolines, *Tetrahedron*, **56** (41), 8197-8205 (2000)

13 Matsuzaki, M., Ellis-Davies, G. C., Nemoto, T., Miyashita, Y., Iino, M., Kasai, H., Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons, *Nature Neuroscience*, **4** (11), 1086-1092 (2001)

14 Givens, R. S., Matuszewski, B., Photochemistry of phosphate esters: an efficient method for the generation of electrophiles, *Journal of the American Chemical Society*, **106** (22), 6860-6861 (1984)

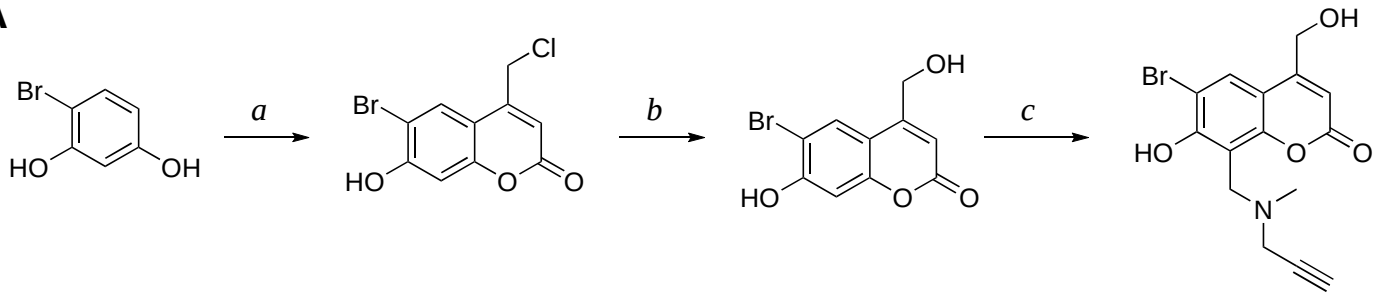
15 Furuta, T., Torigai, H., Sugimoto, M., Iwamura, M., Photochemical Properties of New Photolabile cAMP Derivatives in a Physiological Saline Solution, *Journal of Organic Chemistry*,

60 (13), 3953-3956 (1995)
16 Hagen, V., Frings, S., Wiesner, B., Helm, S., Kaupp, U. B., Bendig, J., [7-(Dialkylamino)coumarin-4-yl]methyl-Caged Compounds as Ultrafast and Effective Long-Wavelength Phototriggers of 8-Bromo-Substituted Cyclic Nucleotides. *ChemBioChem*, **4** (5), 434-442 (2003)
17 Momotake, A., Lindegger, N., Niggli, E., Barsotti, R. J., Ellis-Davies, G. C., The nitrodibenzofuran chromophore: a new caging group for ultra-efficient photolysis in living cells. *Nature Methods*, **3** (1), 35-40 (2006)
18 Specht, A., et al., New photoremovable protecting groups for carboxylic acids with high photolytic efficiencies at near-UV irradiation. Application to the photocontrolled release of L-glutamate. *ChemBioChem*, **7** (11), 1690-1695 (2006)
19 Petersen, S., Alonso, J. M., Specht, A., Duodu, P., Goeldner, M., del Campo, A., Phototriggering of cell adhesion by caged cyclic RGD peptides. *Angewandte Chemistry International Edition*, **47** (17), 3192-3195 (2008)
20 Heckman, L. M., et al., Design and Synthesis of a Calcium-Sensitive Photocage. *Angewandte Chemistry International Edition*, **55** (29), 8363-8366 (2016)
21 Olson, J. P., Banghart, M. R., Sabatini, B. L., Ellis-Davies, G. C., Spectral Evolution of a Photochemical Protecting Group for Orthogonal Two-Color Uncaging with Visible Light, *Journal of the American Chemical Society*, **135** (42), 15948-15954 (2013)
22 Gandioso, A., et al., Sequential Uncaging with Green Light can be Achieved by Fine-Tuning the Structure of a Dicyanocoumarin Chromophore, *ChemistryOpen*, **6** (3), 375-384 (2017).
23 Bassolino, G., Nancoz, C., Thiel, Z., Bois, E., Vauthey, E., Rivera-Fuentes, P., Photolabile coumarins with improved efficiency through azetidinyl substitution. *Chemical Science*, **9** (2), 387-391 (2018)
24 Lin, Q. N., et al., Coumarin Photocaging Groups Modified with an Electron-Rich Styryl Moiety at the 3-Position: Long-Wavelength Excitation, Rapid Photolysis, and Photobleaching. *Angewandte Chemistry International Edition,* **57** (14), 3722-3726 (2018)
25 Nani, R. R., et al., In Vivo Activation of Duocarmycin-Antibody Conjugates by Near-Infrared Light. *ACS Central Science*, **3** (4), 329-337 (2017)
26 Umeda, N., et al. Boron Dipyrromethene As a Fluorescent Caging Group for Single-Photon Uncaging with Long-Wavelength Visible Light. *ACS Chemical Biology*, **9** (10), 2242-2246 (2014)
27 Slanina, T., et al., In Search of the Perfect Photocage: Structure–Reactivity Relationships in meso-Methyl BODIPY Photoremovable Protecting Groups. *Journal of the American Chemical Society*, **139** (42), 15168-15175 (2017)
28 Furuta, T., et al., Brominated 7-hydroxycoumarin-4-ylmethyls: Photolabile protecting groups with biologically useful cross-sections for two photon photolysis. *Proceedings of the National Academy of Sciences of the United States of America* **96** (4), 1193-1200 (1999).
29 Furuta, T., et al., Bhc-cNMPs as either water-soluble or membrane-permeant photoreleasable cyclic nucleotides for both one- and two-photon excitation. *ChemBioChem.* **5** (8), 1119-1128 (2004).
30 Suzuki, A. Z., et al., Coumarin-4-ylmethoxycarbonyls as phototriggers for alcohols and phenols. *Organic Letters* **5** (25), 4867-4870 (2003).
31 Ando, H., Furuta, T., Tsien, R. Y., Okamoto, H., Photo-mediated gene activation using caged RNA/DNA in zebrafish embryos. *Nature Genetics* **28** (4), 317-325 (2001).
32 Teraoka, A., Murakoshi, K., Fukamauchi, K., Suzuki, A. Z., Watanabe, S., Furuta, T., Preparation

and affinity-based purification of caged linear DNA for light-controlled gene expression in mammalian cells. *Chemical Communications* **50** (6), 664-666 (2014).
33 Watanabe, T., et al., Synthesis of nucleobase-caged peptide nucleic acids having improved photochemical properties. *Organic and Biomolecular Chemistry* **12** (28), 5089-5093 (2014).
34 Horinouchi, T., Nakagawa, H., Suzuki, T., Fukuhara, K., Miyata, N., A novel mitochondria-localizing nitrobenzene derivative as a donor for photo-uncaging of nitric oxide. *Bioorganic & Medicinal Chemistry Letters*, **21** (7), 2000-2002 (2011)
35 Leonidova, A., et al., Photo-induced uncaging of a specific Re(I) organometallic complex in living cells. *Chemical Science*, **5** (10), 4044-4056 (2014)
36 Nadler, A., et al., Exclusive photorelease of signalling lipids at the plasma membrane. *Nature Communications*, **6**, 10056 (2015)
37 Feng, S. H., et al., Mitochondria-specific photoactivation to monitor local sphingosine metabolism and function. *Elife*, **7**, e34555 (2018)
38 Wagner, N., Stephan, M., Hoglinger, D., Nadler, A., A Click Cage: Organelle-Specific Uncaging of Lipid Messengers. *Angewandte Chemistry International Edition* **57** (40), 13339-13343 (2018).
39 Feng, S., Harayama, T., Chang, D., Hannich, J. T., Winssinger, N., Riezman, H., Lysosome-targeted photoactivation reveals local sphingosine metabolism signatures. *Chemical Science*, **10** (8), 2253-2258 (2019)
40 Furuta, T., Manabe, K., Teraoka, A., Murakoshi, K., Ohtsubo, A., Suzuki, A., Design, synthesis, and photochemistry of modular caging groups for photoreleasable nucleotides. *Organic Letters* **14** (24), 6182-6185 (2012).
41 Suzuki, A. Z., et al., A clickable caging group as a new platform for modular caged compounds with improved photochemical properties. *Chemical Communications* **55** (4), 451-454 (2019).
42 Hatchard, C. G., Parker, C. A., A new sensitive chemical actinometer - II. Potassium ferrioxalate as a standard chemical actinometer, *Proceedings of the Royal Society A*, **235** (1203), 518-536 (1956)
43 Furuta, T., Nishiyama, K., Manabe, A., Fukuoka, M., Iwamura, M, Design, synthesis and photochemical properties of caged compounds of lipid mediators, *Proceedings of the ISBC 2003*, 124-125 (2003).
44 Shirai, Y., Segawa, S., Kuriyama, M., Goto, K., Sakai, N., Saito, N., *The Journal of Biological Chemistry*, **275** (32), 24760-24766 (2000)
45 Furuta, T., Noguchi, K., Controlling cellular systems with Bhc-caged compounds. *TrAC, Trends in Analytical Chemistry.* **23** (7), 511-519 (2004).
46 Furuta, T., Designing caged compounds for spatiotemporal control of cellular chemistry. *Journal of the Synthetic Organic Chemistry Japan.* **69** (11), 1164-1169 (2012).
47 Furuta, T., Coumarin-4-ylmethyl Phototriggers. In *Dynamic Studies in Biology: Phototriggers, Photoswitches and Caged Biomolecules*, Goeldner, M.; Givens, R. S., Eds. 29-55, WILEY-VCH (2005).
48 Furuta, T., Watanabe, T., Tanabe, S., Sakyo, J., Matsuba, C., Phototriggers for Nucleobases with Improved Photochemical Properties. *Organic Letters* **9** (23), 4717-4720 (2007).
49 Manova, R., van Beek, T. A., Zuilhof, H., Surface Functionalization by Strain-Promoted Alkyne–Azide Click Reactions. *Angewandte Chemistry International Edition* **50** (24), 5428-5430 (2011).
50 Adams, S. R., Kao, J. P. Y., Grynkiewicz, G., Minta, A., Tsien, R. Y., Biologically Useful Chelators

That Release Ca2+ Upon Illumination. *Journal of the American Chemical Society* **110** (10), 3212-3220 (1988).

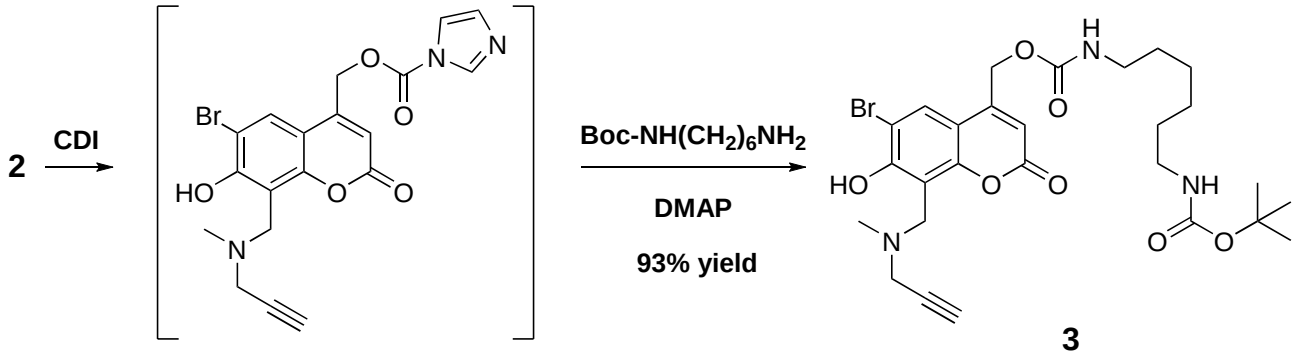
A



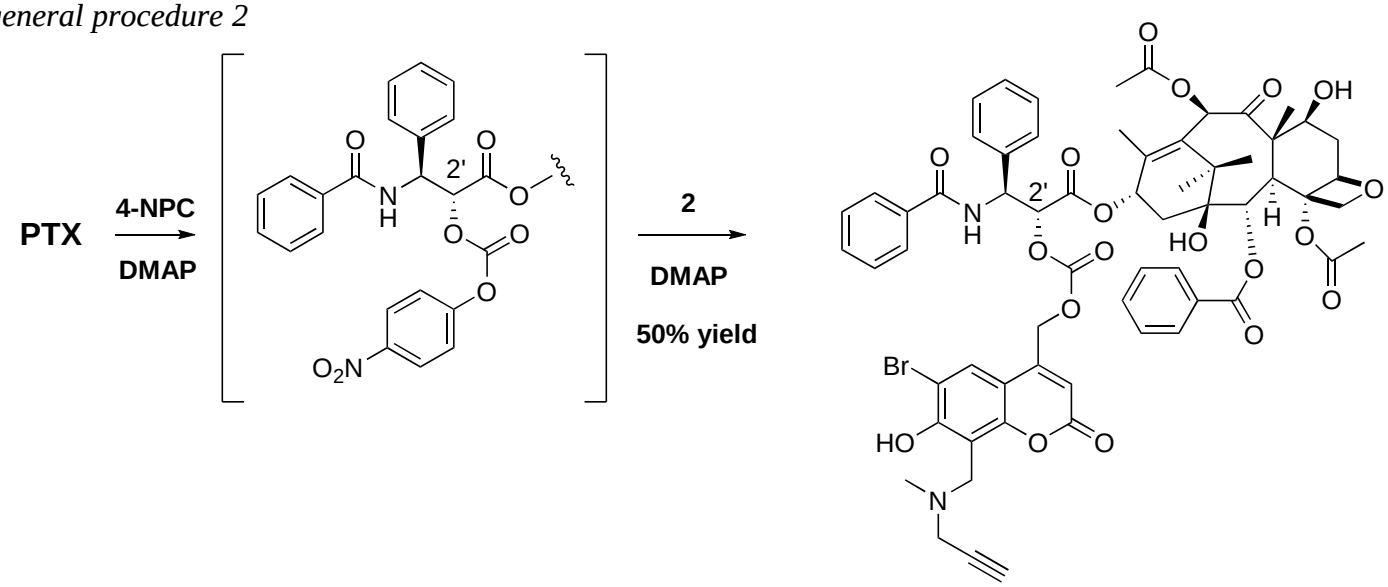
BhcCH$_2$OH (1) **paBhcCH$_2$OH (2)**

B

general procedure 1

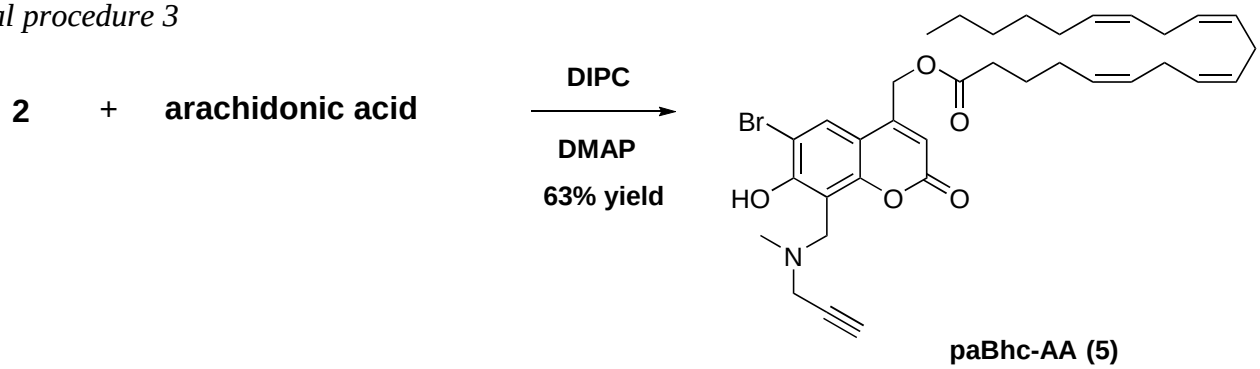


general procedure 2



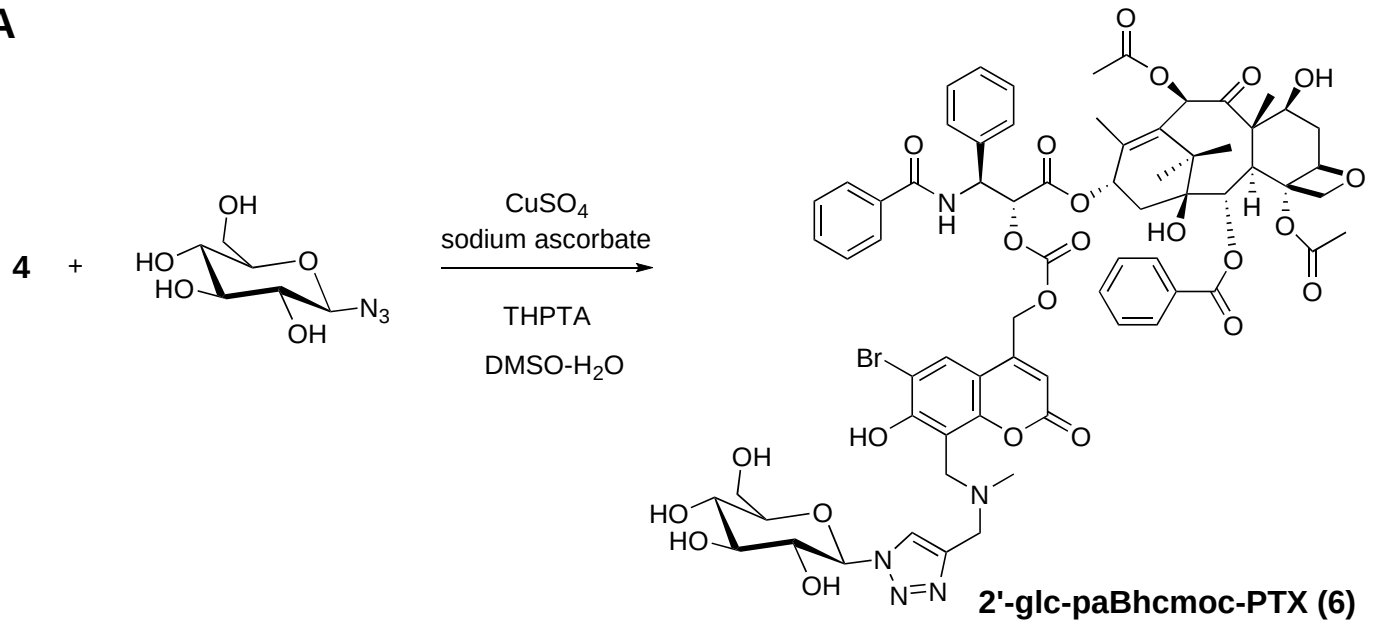
2'-paBhcmoc-PTX (4)

general procedure 3



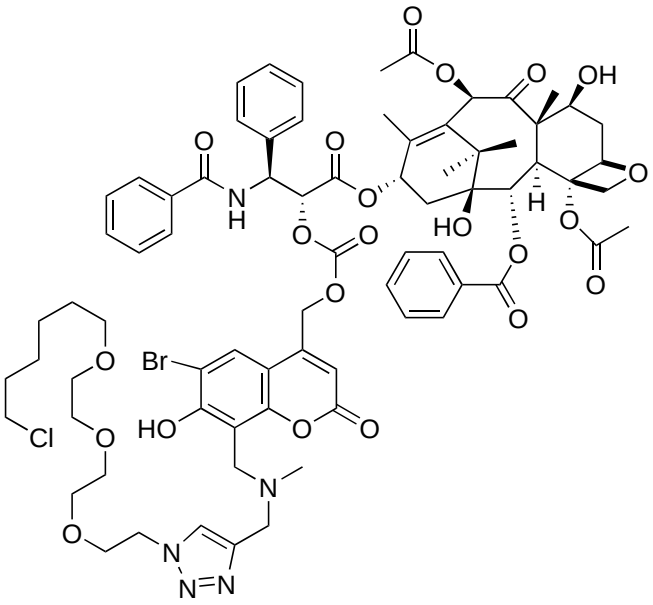
paBhc-AA (5)

A

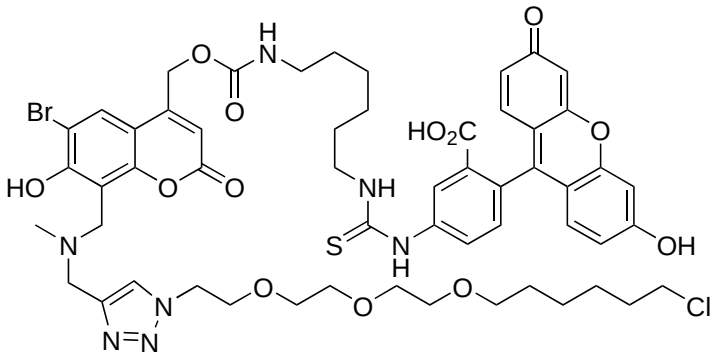


2'-glc-paBhcmoc-PTX (6)

B



2'-Halo-paBhcmoc-PTX (7)



paBhc-hex-FITC/Halo (8)

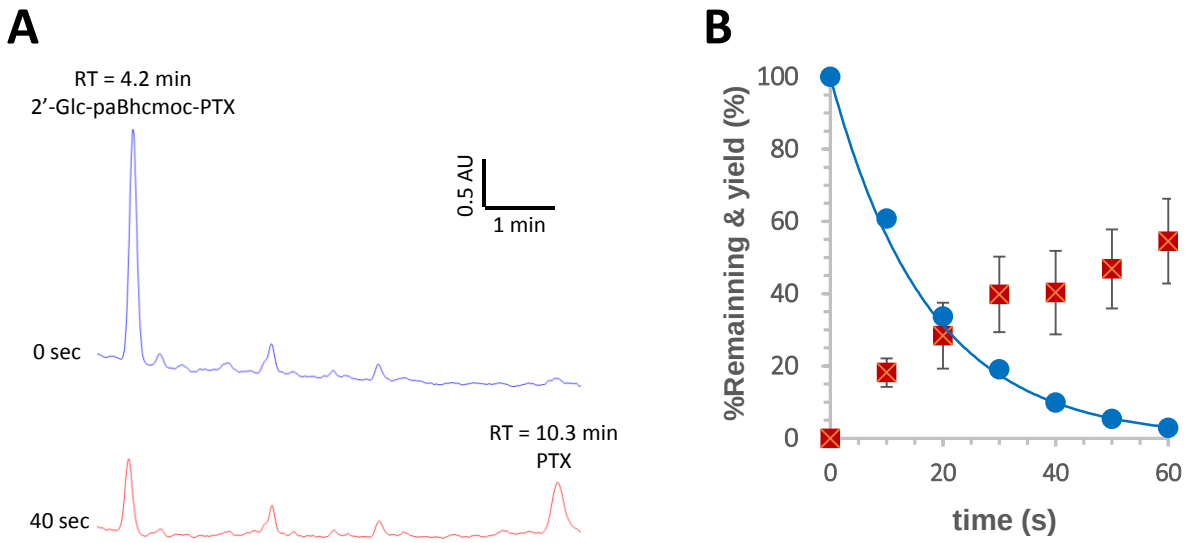


Figure4_tfuruta

Click here to access/download;Figure;Fig4_tfuruta.pdf

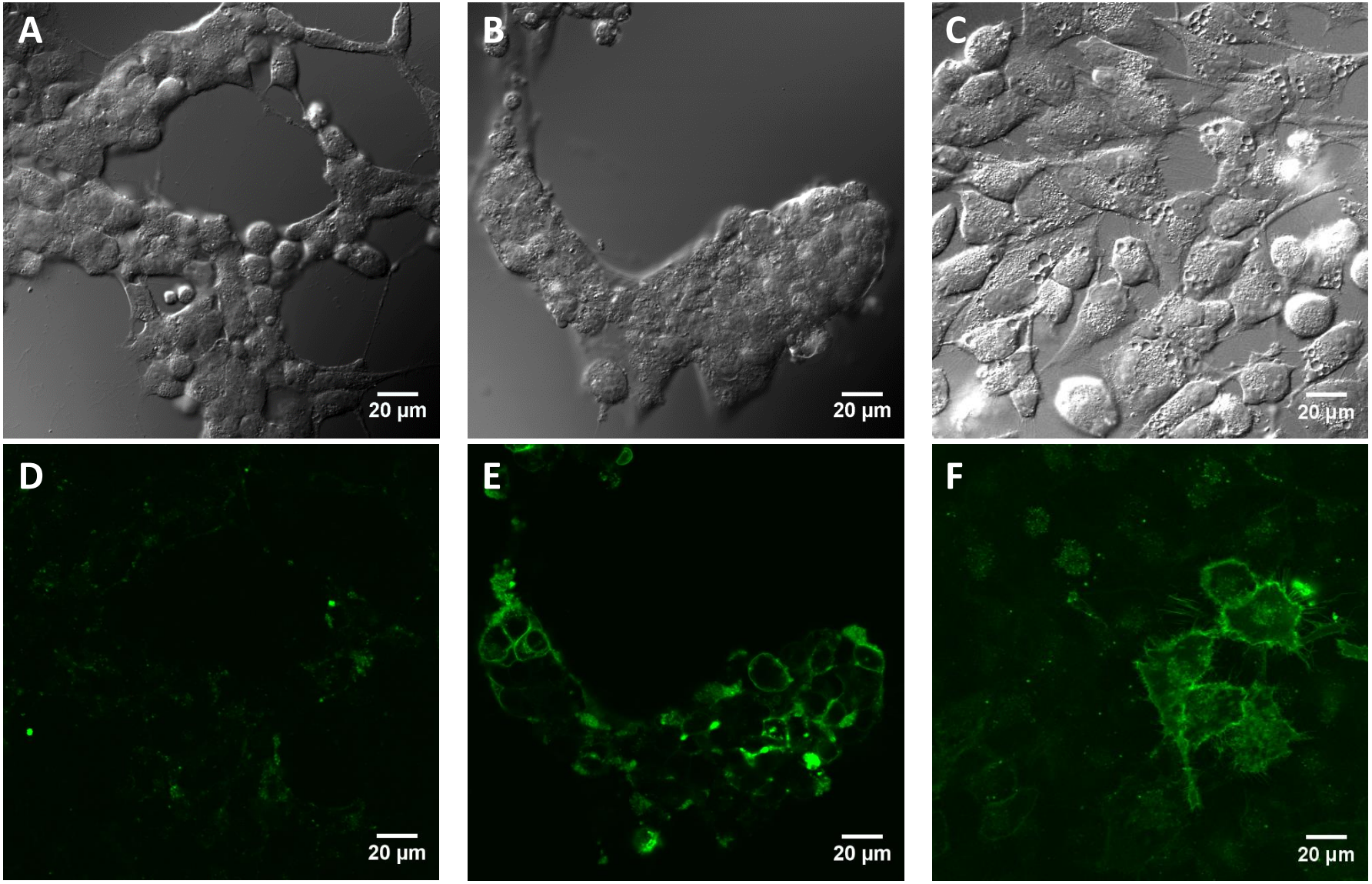
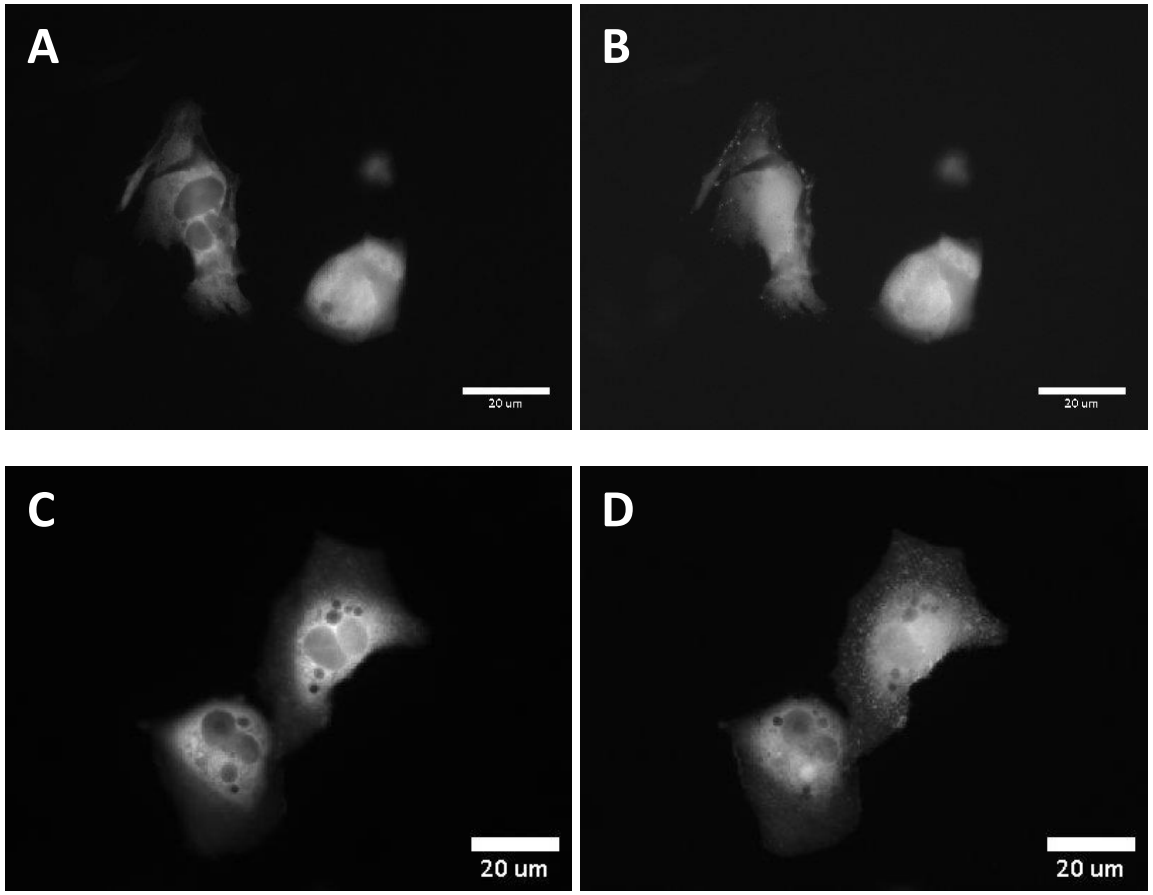


Figure5_tfuruta



Table

table 1

compounds	λ_{max} (nm)[a]	ε_{max} (M^{-1} cm^{-1})[b]	Φ_{dis}[c]	$\varepsilon\Phi_{dis}$[d]	Solubility (μM)[e]
PTX					1.0
2'-Bhcmoc-PTX	340	10500	0.040	400	55
2'-paBhcmoc-PTX	359	9300	0.059	670	8.3
2'-glc-Bhcmoc-PTX	373	12300	0.14	1280	650
Bhc-AA	341	10800	0.038	390	
paBhc-AA	366	10300	0.083	750	

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_tf.xls

Name of Material/ Equipment	Company	Catalog Number
acetonitrile, EP	Nacalai	00404-75
acetonitrile, super dehydrated	FUJIFILM Wako	010-22905
Antibiotic-Antimycotic, 100X	Thermo Fisher	15240062
4-bromoresorcinol	TCI Chemicals	B0654
N,N'-carbonyldiimidazole	FUJIFILM Wako	034-10491
chloroform	Kanto	07278-71
Copper (II) Sulfate Pentahydrate, 99.9%	FUJIFILM Wako	032-12511
dichloromethane, dehydrated	Kanto	11338-05
N,N '-Diisopropylcarbodiimide (DIPC)	TCI Chemicals	D0254
4-dimethylaminopyridine	TCI Chemicals	D1450
dimethylsulfoxide, dehydrated -super-	Kanto	10380-05
DMEM - Dulbecco's Modified Eagle Medium	Sigma	D6046-500ML
dual light source fluorescence illuminator, IX2-RFAW	Olympus	
Ethanol (99.5)	FUJIFILM Wako	054-07225
Ethyl 4-Chloroacetoacetate	TCI Chemicals	C0911
Ham's F-12 with L-Glutamine and Phenol Red	FUJIFILM Wako	087-08335
hydrochloric acid	FUJIFILM Wako	087-01076
inverted fluorescent microscope IX-71	Olympus	
ISOLUTE Phase Separator, 15 mL	Biotage	120-1906-D
L-(+)-Ascorbic Acid Sodium Salt	FUJIFILM Wako	196-01252
laser scanning fluorescence confocal microscopy, FLUOVIEW FV1200/IX-81	Olympus	
Lipofectamine 2000 Transfection Reagent	Thermo Fisher	11668027
3-(*N* -morpholino)propanesulfonic acid	Dojindo	345-01804
4-nitrophenylchloroformate (4-NPC)	TCI Chemicals	C1400
Opti-MEM I Reduced Serum Medium, no phenol red	Thermo Fisher	11058021
1,10 - Phenanthroline Monohydrate	Nacalai	26707-02
Photochemical reactor with RPR 350 nm lamps	Rayonet	
Potassium Trioxalatoferrate (III) trihydrate	FUJIFILM Wako	W01SRM19-5000
Sodium Acetate Trihydrate	Nacalai	31115-05
Sodium Bicarbonate	FUJIFILM Wako	199-05985
Sulfuric Acid, 96-98%	FUJIFILM Wako	190-04675
Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA)	ALDRICH	762342-100MG
tri - Sodium Citrate Dihydrate	Nacalai	31404-15

Xenon light source, MAX-303

Asahi Spectra

Comments/Description

lipofection reagent
MOPS

reduced serum medium contains no phenol red

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Design, synthesis, and photochemical properties of clickable caged compounds

Author(s): A.Z. Suzuki, Y. Shiraishi, H. Aoki, H. Sasaki, R. Watahiki, T. Furuta

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Toshiaki Furuta
Department:	Department of Biomolecular Science
Institution:	Toho University
Title:	Professor
Signature:	Toshi Furuta
Date:	June 15, 2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Toho University

Department of Biomolecular Science
Faculty of Science, Toho University
2-2-1 Miyama, Funabashi, Chiba, 274-8510, Japan
Tel. +81-47-472-1169 Fax. +81- 47-472-1169
furuta@biomol.sci.toho-u.ac.jp

Dr. Toshiaki Furuta
Professor of Biomolecular Science

June 15, 2019

Dr. Bing Wu
Review Editor
JoVE

Dear Dr. Wu,

Please find enclosed our revised manuscript "Design, synthesis, and photochemical properties of clickable caged compounds" with the Manuscript ID: JoVE60021. This contribution was submitted on the 21st of March and was returned for revision on May 13th.

Based on the four reviewers' useful and valid suggestions and comments, we have done additional experiments and made several changes to address the points raised by the reviewers. In the following pages, please find detailed responses to the reviewers' comments.

Yours sincerely,

Toshiaki Furuta
Professor of Biomolecular Science

Toho University

Revisions made to respond to the Editorial Comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Figure 3: Please define error bars in the figure legend. Please explain what different colors represent.
3. Figure 4: Please include a space between numbers and its corresponding unit of the scale bar (i.e., 20 µm).
4. References: Please do not abbreviate journal titles.
5. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Our answers

1. The manuscript has been edited by a native speaker of English. We would like to thank Editage (www.editage.jp) for English language editing.
2. The definition of error bars and the explanation of plots have been added in the figure legend.
3. The description of the scale bars has been corrected.
4. The journal titles have been spelled out in full.
5. The items are listed in alphabetical order.
6. I have added Yukiko Shiraishi as a co-author. She is responsible for the additional experiments described in Figure 5.
7. The following material and equipment have been added to the materials sheet.
   dual light source fluorescence illuminator, IX2-RFAW,
   Ham's F-12 with L-Glutamine and Phenol Red
   inverted fluorescent microscope IX-71

Please find below detailed responses to the editorial comments. The text that was not highlighted has been rewritten, but the content has not been changed. The contents of the yellow highlighted text have been added to the revised manuscript.

	Revised	**Primary submitted**
2	**Figure 3: Photolysis of 2'-glc-paBhcmoc-PTX (6)**. Samples (10 µM) in K-MOPS solutions (pH 7.2) were irradiated at 350 nm. (**A**) Typical HPLC traces for the photolysis of 6 (measured at 254 nm). Samples were analyzed at the specified irradiation time. (**B**) Time course for the photolysis of **6**. Blue circles show the consumption of **6**. The solid line shows the least-squares curve fit for a simple decaying exponential for **6**. Red squares show the yield of PTX. The error bars represent the standard deviation (±SD).	**Figure 3: Photolysis of 2'-glc-paBhcmoc-PTX (6)**. Samples (10 µM) in K-MOPS solutions (pH 7.2) were irradiated at 350 nm. (**A**) Typical HPLC traces for the photolysis of 6 (measured at 254 nm). Samples were analyzed at the specified irradiation time. (**B**) Time course for the photolysis of **6**.
7		

Improvements and changes made to respond to Reviewers comments:

Reviewer #1

Furuta and coworkers report the synthesis of clickable caged compounds and their photochemical properties. The paper is well-written and -described for most readers of this visualized journal. I have a just small correction on this paper, but, the correction would be possible during the final proof reading duration.
Φ in Table should be Φdis.

Our answers

We thank the reviewer for the encouraging and helpful comments to improve the manuscript. We changed Φ to Φ_{dis} in Table 1.

Reviewer #2

Major Concerns:
However, the authors suggest to focus the main part of their video on the chemical synthesis and characterization of caged compounds. This in itself does not seem to be very useful: Organic synthesis is a very well-established method and there are literally tens of thousands of chemists that are more than able to make a molecule from published (written) protocols. On the other hand, biologists will not suddenly start synthesizing probes - primarily because the necessary equipment & expertise

does not exist in their labs.
The actual application of caged compounds is limited to three sub-points in the protocol (5.6-5.8) and there is no actual uncaging experiment included. I recommend to significantly strengthen this aspect according to the following points:

- What are proper controls in uncaging experiments, and how are they best implemented?
- Which microscopy equipment is required?
- What are useful readouts? (examples of biosensors, fluorescent proteins, calcium and so on)
- Which type of processes & time-scales can be assessed?
- For what kind of biological questions is this approach useful?
- What are the advantages with regard to other techniques (optogenetics, chemical dimerizers etc.)

If the authors would agree proceed to implement these aspects & shorten the organic synthesis aspects, I'd be more then happy to support their submission. In the present form, I do not think that the proposed video fits with what JoVE is trying to do.
Minor Concerns:
There are few other points that need cleaning up.
1. Citation habits. 14 out of 20 literature citations refer to papers that were co-authored by the main author, and most of the other references are overview articles, not original work. Citing a few key examples of one's own work to provide proper context for the current work is fine & even required, but this goes a bit too far. There are numerous people working on caged compounds, and there are quite a few examples of organelle targeted caged compounds. Besides the example from the Nadler lab that the authors did cite, there is earlier work from the Miyada lab (Bioorg. Med. Chem. Lett. 2011), Gasser lab (Chem. Sci. 2014), Schultz lab (Nat. Commun. 2015) and Riezman lab (Elife 2017 and Chem. Sci. 2019).
2. Figure 3: The chromatograms contain numerous peaks that appear to be something else, as they do not change upon illumination. The authors should make sure that only pure compounds are used.
3. Figure 4 contains panels A-F, but the figure legend is only A-D. It is thus not clear what control & transfected cells are. It would also be nice if there would be an independent control for transfection apart from the stain induced by the compound to allow for colocalization analysis

Our answers

We thank the reviewer for the valuable and helpful comments to improve the manuscript. Based on the reviewer's suggestions, we have made the following additions and modifications.
Major Concerns:

a) An example of a live cell uncaging experiment of one of the clickable caged compounds described in our previous paper (ref. 41) has been added to Fig. 5 and protocols 6.1-6.7. Description of the additional experiments has also been added to the representative results section. The experiment was performed using standard, inverted fluorescence microscopy equipped with a double lamphouse unit as described in protocol 6 and the materials sheet. A fluorescent protein tag such as EGFP can be used as a readout for the experiments.
b) Protocols for video presentation have been changed. Protocols 1.3 (organic syntheses) have been removed, while 6.2-6.7 (live cell uncaging) have been added.
c) The appropriate equipment and readouts for live cell uncaging experiments, the type of biological processes to be manipulated, and the biological questions to be addressed vary from molecule to molecule. I agree that what the reviewer pointed out is particularly important when submitting new experimental results and concepts on caged compounds. My understanding of the focus of the journal is to provide a step-by-step method of previously reported experiments. In addition, the main purpose of our manuscript is to expand the repertoire of caging groups and to provide a method for the preparation of clickable platforms for caged compounds that can be modified easily with additional properties. Based on this line, we have shown three representative results of the use of clickable caged compounds (Table 1, Fig. 4 and Fig. 5). Taking into account the above points, we added additional experiments described in Figure 5 to respond to the comments of the reviewer and would prefer not to mention some of the points that the reviewer have pointed out.

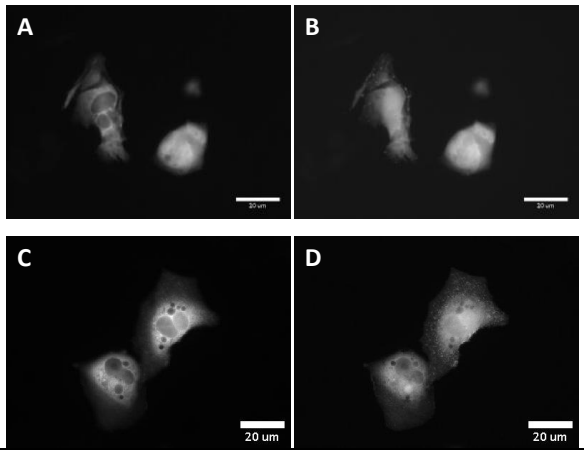
Minor Concerns:

1) I realize that our original citations are not properly addressed to the work of other groups. We have cited several additional references, including review articles on caged compounds (ref. 1, 5 and 6), studies on the development of caging groups (7-27), and earlier work of organelle targeted caged compounds (34-37, 39).
2) We used KMops buffer (consisted of 100 mM KCl/10 mM Mops titrated to pH 7.2 with KOH) containing 0.1% DMSO as a solvent. Most of the small peaks other than 2'-glc-paBhcmoc-PTX

and PTX in the HPLC traces are found in the solvent itself.

3) I apologize for the missing of the figure legends in Figure 4. I have added the statement describing the panels E and F.

Please find below detailed responses to the reviewer's comments. The text that was not highlighted has been rewritten, but the content has not been changed. The contents of the grey highlighted text have been removed and the yellow highlighted text has been added to the revised manuscript.

	Revised	**Primary submitted**
a)	In the main text In live cell experiments, the targeting of paBhc-hex-FITC/Halo to the cultured mammalian cells transiently expressing a fusion protein of a HaloTag protein and epidermal growth factor receptor (EGFR) was achieved successfully. Green fluorescence of the fluorescein moiety of paBhc-hex-FITC/Halo was observed on the cell membrane (Figure 4). Photo-mediated modulation of the subcellular localization of a kinase was achieved using a paBhc caged compound. The translocation of diacylglycerol kinase γ (DGKγ) has been reported to be activated in the presence of arachidonic acid (AA)[44]. CHO-K1 cells transiently expressing GFP-DGKγ were treated with either AA or paBhc-AA (5). Addition of AA caused the modulation of the subcellular localization of DGKγ (Fig. 5 (A) and (B)). Similar changes in the localization of DGKγ were observed for the paBhc-AA-treated cells after exposure to UV light (Fig. 5 (C) and (D)).	In the main text In live cell experiments, targeting of paBhc-hex-FITC/Halo to HeLa cells transiently expressing a fusion protein of a HaloTag protein and EGFR was achieved successfully. Green fluorescence of the fluorescein moiety of paBhc-hex-FITC/Halo was observed on the cell membrane (Figure 4).
	Figure 5  	not investigated
	Figure 5: Fluorescence images after UV irradiation of CHO-K1 cells incubated with Bhc caged arachidonic acid. CHO-K1 cells were transfected with a fusion protein DGKγ-EGFP. (A) A fluorescence image of the transfected cells. (B) 100 s after the addition of a 10 μM solution of arachidonic acid. (C) Cells were incubated with a 10 μM solution of paBhc-AA (5) at 37 °C for 5 min. (D) 100 s after 20-s UV irradiation (330–385 nm).	not investigated
	6. Photomediated modulation of a kinase localization using a clickable caged compound Prior to use, maintain the CHO-K1 cells in Ham's F-12 medium supplemented with 10% FBS at 37 °C and 5% CO2. 6.1. Prepare a 100× working solution (1 mM)of paBhc-AA (5) in DMSO. NOTE: A 10 mM stock solution of the compound is prepared and stored in a freezer (-20 °C).	

	6.2. Seed approximately 5 × 105 cells per dish into 35 mm glass bottom dishes in DMEM (2 mL) 24 h before transfection. 6.2. Transfect CHO-K1 cells with a plasmid coding for GFP-DGKγ 48 h before the uncaging experiments. NOTE: Transfection is performed according to protocols 5.2–5.5. 6.3. Replace the medium with a reduced serum medium (2 mL). Add the 100× paBhc-AA working solution (20 µL) and incubate the cells at 37 °C and 5% CO2 for between 5 min and 1 h. NOTE: The loading time depends on the compound employed. 6.4. Place the cells on the objective stage of an inverted fluorescent microscope equipped with a dual light source fluorescence illuminator. 6.5. Take a fluorescent image every 10 s. Irradiate the cells with 330–385 nm light through a microscope objective for an appropriate time. Alternatively, irradiate the cells with 405 nm light using a Xe lamp through flexible quartz fibers. 6.6. Continue to record fluorescent images for 10 min.	
3)	**Figure 4: Fluorescence images of cultured mammalian cells incubated with paBhc-hex-FITC/Halo (8).** Cells transfected with pcDNA3-Halo-EGFR were incubated with a 2 µM solution of compound 8 at 37 °C for 30 min. The images were obtained after repeated washing with PBS+. Mock-treated HEK293T cells ((A) differential interference contrast (DIC) image and (D) fluorescence image). HEK293T cells ((B) and (E)) and HeLa cells ((C) and (F)) transiently expressing Halo-EGFR ((B) and (C): DIC images and (E) and (F): fluorescence images).	**Figure 4: Fluorescence images of HeLa cells incubated with paBhc-hex-FITC/Halo (8).** Cells transfected with pcDNA3-Halo-EGFR were incubated with 2 µM compound 8 at 37 °C for 30 min. The images were taken after repeated washing with PBS+. Mock treated cells ((A) differential interference contrast (DIC) image and (B) fluorescence image) and cells transiently expressing Halo-EGFR ((C) DIC image and (D) fluorescence image).

Reviewer #3

Major Concerns:

It would be necessary to describe more clearly the definition and introduction of the concept of caged compound and its advantages over other techniques where light is used as an external agent for therapy.

Minor Concerns:

1) There are multiple typos that need attention before publication. Improvement of sentence structure and grammar is needed in some cases. (e.g. line 44 and 45)
2) Little consistency in the values described in the protocols section, decimals should be homogenized. (e.g. 1.2 and 1.3 and 2.1)
3) In the method of the click reaction, why didn't prepare first the Cu(I) complex and then added over the Azido and the last step over the alkynyl?
4) It would be appropriate to mention of yields even if it is the description of the methods (and introduce in the scheme)
5) The units in the table it's necessary.
6) It would be appropriate to improve the scientific vocabulary in general, both at a chemical and biological level, because we must speak properly in some cases. (5 section)
7) The bibliography used should be reviewed, because many examples of the literature are missing and also in the field of caged compounds, (Prof. Heckel, Dr. Marchan, Dr. Schnermann, Dr. Rivera-Fuentes, Dr.Lavis, Dr. delCampo ...)

Our answers

We thank the reviewer for the encouraging and helpful comments to improve the manuscript. Based on the reviewer's suggestions, we have made the following additions and modifications.

Major Concerns:

a) We added sentences which add more explanations about caged compounds in the Introduction section.

Minor Concerns:

1) The manuscript has been edited by a native speaker of English as mentioned above.
2) The significant figures of the amount of substance in sections 1.1, 1.2, 1.3, and 2.1 have been homogenized to three digits.
3) There are several protocols for Cu (I) catalyzed Huisgene reaction. The premixing of CuSO4 and sodium ascorbate to produce Cu (I) species first is one of the examples. Other methods include the addition of CuSO4 and ascorbate to the mixture of an alkyne and an azide.
4) The isolated yields of the products have been mentioned in Figure 1, the figure legend and

protocol 2.

5) In the original manuscript, the unit of measurement is described as a table footnote. We have also added units to the table, following the reviewer's suggestion.

7) We have added thirty references, including the literature that the reviewer suggested (ref. 1, 11, 19, 20, 22, 23, 25).

Please find below detailed responses to the reviewer's comments. The text that was not highlighted has been rewritten, but the content has not been changed. The contents of the grey highlighted text have been removed and the yellow highlighted text has been added to the revised manuscript.

	Revised	**Primary submitted**
a)	Caged compounds are designed synthetic molecules whose original functions are temporally masked by covalently attached photo-removable protecting groups. Interestingly, caged compounds of biologically relevant molecules provide an indispensable method for the spatiotemporal control of the cellular physiology1–6. In 1977, Engels and Schlaeger reported the 2-nitrobenzyl ester of cAMP as a membrane permeable and photolabile derivative of cAMP7. The following year, Kaplan reported the 1-(2-nitrophenyl)ethyl ester of ATP (NPE-ATP) and named this compound "caged" ATP8. Since then, a range of photochemically removable protecting groups such as 2-nitrobenzyls, p-hydroxyphenacyls9, 2-(2-nitrophenyl)ethyls10,11, nitroindolins12,13, and (coumarin-4-yl)methyls14–16 have been used for the preparation of caged compounds. The synthesis of caged compounds with desirable additional properties such as membrane permeability, water solubility, and cellular targeting ability would be expected to facilitate cell biological applications. Since the physical and photochemical properties of these molecules depend primarily on the chemical structure of the photochemically removable protecting groups used to prepare them, a diverse repertoire of photo-caging groups is required. However, the structural diversity of currently available caging groups that exhibit high photolysis efficiencies is limited. This could be an obstacle to increasing the use of caged compounds. To address this issue, the repertoire of photo-caging groups has been expanded by the chemical modification of existing photoremovable protecting groups or the design of new photolabile chromophores with superior photophysical and photochemical properties. Examples include nitrodibenzofuran (NDBF)17, [3-(4,5-dimethoxy-2-nitrophenyl)-2-butyl] (DMNPB)18,19, a calcium-sensitive 2-nitrobenzyl photocage20, substituted coumarinylmethyls (DEAC45021, DEAdcCM22, 7-azetidinyl-4-methylcoumarin23, and styryl coumarins24), cyanine derivatives (CyEt-pan)25, and BODIPY derivatives26,27. In addition, we previously developed the (6-bromo-7-hydroxycoumarin-4-yl)methyl (Bhc) group and successfully synthesized various caged compounds of neurotransmitters28, second messengers29,30, and oligonucleotides31–33 exhibiting large one- and two-photon excitation cross-sections. If additional properties can be installed easily into the Bhc group without compromising its photosensitivity, then the repertoire of caged compounds can be expanded34–39. We therefore designed modular caged	Caged compounds are designed synthetic molecules whose original functions are temporally masked by covalently attached photo-removable protecting groups1–3. Caged compounds of biologically relevant molecules provide an indispensable method for spatiotemporal control of cellular physiology. We have developed caged compounds of neurotransmitters4, second messengers5,6, and oligonucleotides7–9 with large one- and two-photon excitation cross-sections. The synthesis of caged compounds with desirable additional properties such as membrane permeability, water solubility, and cellular targeting ability will facilitate cell biological applications. Since the physical and photochemical properties of the molecules depend primarily on the chemical structure of the photo-chemically removable protecting group (photo-caging group) used to prepare them, a diverse repertoire of photo-caging groups is required. However, the structural diversity of the currently available caging groups with high photolysis efficiencies is limited. This could be the obstacle to increasing the usefulness of caged compounds. If an additional property can be installed easily in photo-caging groups without compromising their photosensitivities, then the repertoire of caged compounds can be expanded10. To this end, we designed modular caged compounds that comprise three parts: photo-responsive cores, chemical handles for installation of additional functionalities, and molecules to be masked11,12.

<table>
<tr><td></td><td>compounds that comprise three parts, namely the Bhc group as a photo-responsive core, chemical handles for the installation of additional functionalities, and the molecules that are to be masked40,41.</td><td></td></tr>
<tr><td>2)</td><td>1.1.8. Dry the precipitate under vacuum overnight to yield BhcCH2Cl as a light brown powder (13.57 g, 46.9 mmol).
1.2.1. Place the prepared BhcCH2Cl (1.1440 g, 3.95 mmol) and...
1.2.2. Remove the water from the reaction by rotary evaporation under vacuum to yield BhcCH2OH (1) as a light brown powder (1.0359 g, 3.82 mmol, 97% yield).</td><td>1.1.8. Dry the precipitate under vacuum overnight to yield BhcCH2Cl as a light brown powder (13.57 g, 46.87 mmol).
1.2.1 Place 1.1440 g (3.952 mmol) of BhcCH2Cl and...
1.2.2 Remove water via rotary evaporation under vacuum to yield 1.0359 g (3.822 mmol, 97% yield) of BhcCH2OH (1)...</td></tr>
<tr><td>4)</td><td>Figure 1: Preparation of the clickable caged compounds. (A) Reagents and conditions: a. ethyl 4-chloroacetoacetate/conc. H2SO4/rt/7 d/91% yield, b. 1 M HCl/reflux/3 d/97%yield. c. N-methylpropargylamine /HCHO/EtOH, then add (1) and heat at reflux for 17 h/79% yield. (B) Syntheses of the clickable caged amine, PTX, and arachidonic acid.</td><td>Figure 1: Synthesis of clickable caged compounds. (A) Reagents and conditions: a. ethyl 4-chloroacetoacetate / conc. H2SO4/rt/7 d, b. 1 M HCl/reflux/3 d. c. N-methylpropargylamine /HCHO/EtOH, and then 1 and reflux for 17 h. (B) Synthesis of clickable caged amine, PTX, and arachidonic acid.</td></tr>
<tr><td>5)</td><td>Table 1
<table>
<tr><th>compounds</th><th>λ_{max} (nm)[a]</th><th>ε_{max} (M^{-1} cm^{-1})[b]</th><th>Φ_{dis}[c]</th><th>$\varepsilon\Phi_{dis}$[d]</th><th>Solubility (μM)[e]</th></tr>
<tr><td>PTX</td><td></td><td></td><td></td><td></td><td>1.0</td></tr>
<tr><td>2'-Bhcmoc-PTX</td><td>340</td><td>10500</td><td>0.040</td><td>400</td><td>55</td></tr>
<tr><td>2'-paBhcmoc-PTX</td><td>359</td><td>9300</td><td>0.059</td><td>670</td><td>8.3</td></tr>
<tr><td>2'-glc-Bhcmoc-PTX</td><td>373</td><td>12300</td><td>0.14</td><td>1280</td><td>650</td></tr>
<tr><td>Bhc-AA</td><td>341</td><td>10800</td><td>0.038</td><td>390</td><td></td></tr>
<tr><td>paBhc-AA</td><td>366</td><td>10300</td><td>0.083</td><td>750</td><td></td></tr>
</table></td><td>Table 1
<table>
<tr><th>compounds</th><th>λ_{max}[a]</th><th>ε_{max}[b]</th><th>Φ_{dis}[c]</th><th>$\varepsilon\Phi_{dis}$[d]</th><th>Solubility[e]</th></tr>
<tr><td>PTX</td><td></td><td></td><td></td><td></td><td>1.0</td></tr>
<tr><td>2'-Bhcmoc-PTX</td><td>340</td><td>10500</td><td>0.040</td><td>400</td><td>55</td></tr>
<tr><td>2'-paBhcmoc-PTX</td><td>359</td><td>9300</td><td>0.059</td><td>670</td><td>8.3</td></tr>
<tr><td>2'-glc-Bhcmoc-PTX</td><td>373</td><td>12300</td><td>0.14</td><td>1280</td><td>650</td></tr>
<tr><td>Bhc-AA</td><td>341</td><td>10800</td><td>0.038</td><td>390</td><td></td></tr>
<tr><td>paBhc-AA</td><td>366</td><td>10300</td><td>0.083</td><td>750</td><td></td></tr>
</table></td></tr>
</table>

Reviewer #4

Major Concerns:

1) Line 66: "If an additional property can be installed easily in photo-caging groups without compromising their photosensitivities, then…. " should be replaced by "If additional properties can be installed in photo caging groups without compromising on their photosensitivity, then ……" The authors should check the tense of the sentence.
2) Figure 4: The green fluorescence is not visible in the images provided.
3) Did authors conduct any cytotoxicity tests with these compounds? If so, the results should be reported. Additionally, flow cytometry can also be conducted for getting information on various physical characteristics of cell populations.
4) In the results section it has been mentioned that the quantum yields on the as synthesized caged compounds were better than the conventional Bhc caged compounds. Can the authors please mention the details how better were they compared with the conventional Bhc compounds?
5) Please check the manuscript for grammatical errors.
6) The ability to modify the properties using Click chemistry should be explored to tune the fluorescence properties of Bhc compounds.

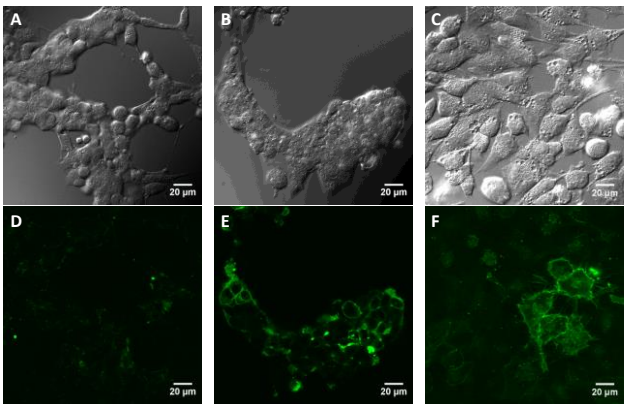
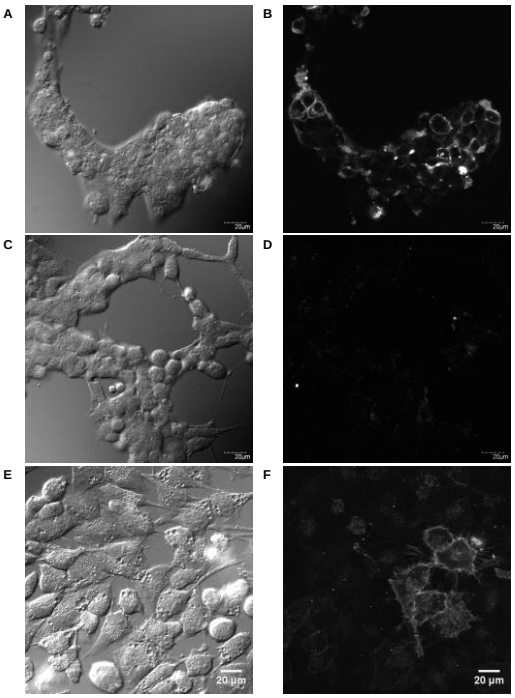
Our answers

We thank the reviewer for the encouraging and helpful comments to improve the manuscript. Based on the reviewer's suggestions, we have made the following additions and modifications.

1) We have revised the sentence.
2) The original fluorescence images were recorded by a monochromatic CCD camera. We have added pseudo-green color to the images D, E, and F in Fig. 4 for clarity. The layout of the figure has also changed.
3) We have not quantified cytotoxicity of the compounds. I agree that cytotoxicity is an important issue. Since the purpose of the present manuscript is to give the readers detailed experimental protocols of the preparation of clickable caged compounds, the experiments proposed by the reviewers are beyond the scope of the paper.
4) We have added values of uncaging quantum yield for clarity.
5) The manuscript has been edited by a native speaker of English as mentioned above
6) Since the clickable handle of the paBhc caging group does not have pi-conjugation with the coumarin ring, we expect that click modification has little effect on the fluorescent properties of the Bhc caging group.

Toho University

Please find below detailed responses to the reviewer's comments. The text that was not highlighted has been rewritten, but the content has not been changed. The contents of the grey highlighted text have been removed and the yellow highlighted text has been added to the revised manuscript.

	Revised	**Primary submitted**
1)	If additional properties can be installed easily into the Bhc group without compromising its photosensitivity, then the repertoire of caged compounds can be expanded[33].	If an additional property can be installed easily in photo-caging groups without compromising their photosensitivities, then the repertoire of caged compounds can be expanded[10].
2)	**Figure 4**  	**Figure 4** 
4)	The quantum yields of clickable caged compounds 2'-glc-paBhcmoc-PTX (Φ_{dis} 0.14) and paBhc-AA (Φ_{dis} 0.083) were more than twice those of conventional Bhc caged compounds 2'-Bhcmoc-PTX (Φ_{dis} 0.040) and Bhc-AA (Φ_{dis} 0.038)[37].	The quantum yields of clickable caged compounds 2'-glc-paBhcmoc-PTX and paBhc-AA were better than those of conventional Bhc caged compounds 2'-Bhcmoc-PTX and Bhc-AA[13].