Electronic Supplementary Information (ESI)

In-cell chemical construction of a photoswitchable CENP-E using a photochromic covalent inhibitor

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1. General Methods

All reagents and solvents were purchased from commercial sources (TCI Chemical, Merck, Kanto Chemical, Fujifilm Wako Pure Chemical Corporation, and Amatek Chemical Co., Ltd.,) and used without further purification. Thin layer chromatography was conducted on silica gel 60 F_{254}-precoated glass plates (Merck). Purification of the synthesized compounds was carried out using Isolera One (Biotage) and a reverse phase high performance liquid chromatography (RP-HPLC) system (Shimadzu). The products were identified using ^{1}H-nuclear magnetic resonance (NMR) spectroscopy with a Biospin AG AV300 spectrometer (BRUKER), and electrospray ionization (ESI) mass spectroscopy with Daltonics microTOF (BRUKER).

Experimental Methods

Experiments for photophysical properties

The UV-Vis absorption spectra of **2**-**4** (20 μM) in the aqueous solution of BRB80 buffer (pH 6.9; 80 mM PIPES, 2 mM $MgCl_2$, 1 mM EGTA) and acetonitrile (1:1) were recorded using a Shimadzu UV-2600 spectrophotometer equipped with temperature controller of TCC-100 with or without light illumination at 365 nm (35 mW cm^{-2}), 385 nm (100 mW cm^{-2}), 405 nm (100 mW cm^{-2}), 430 nm (100 mW cm^{-2}), 450 nm (100 mW cm^{-2}), 505 nm (100 mW cm^{-2}), 525 nm (60 mW cm^{-2}) and 568 nm (60 mW cm^{-2}) using light

sources (CL-1503 with CL-H1-365-9-1, CL-H1-405-9-1, CL-H1-430-9-1, CL-H1-450-9-1, CL-H1-505-9-1, CL-H1-525-7-1 and CL-H1-568-9-1, ASAHI SPECTRA).

mW cm-

Cell culture

HeLa-Kyoto (human cervix adenocarcinoma) cells were cultured in DMEM (Dulbecco's Modified Eagle Medium, with L-glutamine and phenol red, Wako) with or without phenol red supplemented with 10 % FBS (Fetal Bovine Serum, Corning), 1% antibiotic and antimycotic solution (Sigma) at 37 ºC in presence of 5% CO_2 and humidified atmosphere.

Cell viability assay

Cell viability of HeLa cells were evaluated by a colorimetric assay using cell counting kit-8 (CCK-8, Donjido) as the previous report[S1]. Absorbance at 450 nm was measured using the Infinite 200 PRO M Plex plate reader (Tecan). IC_{50} values were estimated by a nonlinear regression analysis (four parameter logistic model), where log[inhibitor] were plotted with the percent of activity.

Counting of cells containing aligned/misaligned chromosomes using microscopic imaging

After six hours of incubation of HeLa cells (1×10^5 cells) with DMEM (+FBS, -phenol red) including **2**-**4** (10 μM) without light illumination, cells were washed with DMEM (+FBS, -phenol red) three times.

For the conditions with light illumination at 365 nm or 568 nm, the cells were incubated for further two hours after light illumination. Then, cells were fixed with 4% PFA (paraformaldehyde) in PBS (phosphate-buffered saline) for 10 min at room temperature. Chromosomes were stained using 1.0 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, Dojindo), overnight at 4 ºC. After washing, the cells were visualized with an Axio Observer 7 microscope (Carl Zeiss) equipped with an Objective Plan-Apochromat 20x/0.8 (Carl Zeiss).

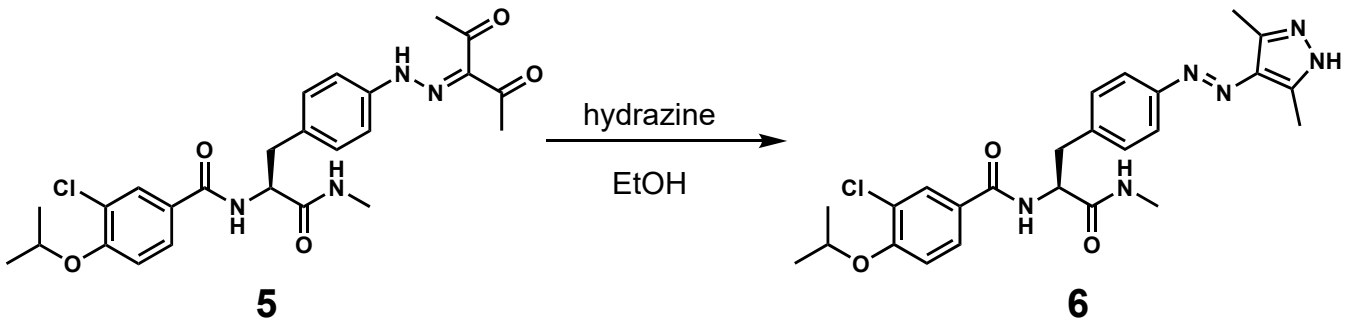
Immunofluorescence experiments

To visualize the localization of chromosomes, CENP-E and mitotic microtubules, immunofluorescence imaging was conducted using the same method as in the previous report[S1]. Briefly, HeLa cells (1×10^5 cells) on a 35 mm glass bottom dish were treated as shown in "**Counting of cells containing aligned/misaligned chromosomes using microscopic imaging**". After methanol fixation, the nuclei were stained with 1.0 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, Dojindo). CENP-E and microtubules were also stained with an anti–α-tubulin antibody (YOL1/34; Abcam) with Alexa Fluor 568-conjugated secondary antibody and the anti-CENP-E antibody (1H12, ab5093, Abcam) with Alexa Fluor 488 secondary antibody. Images were taken with an Axio Observer 7 microscope (Carl Zeiss) equipped with an Objective Plan-Apochromat 63x/1.4 (Carl Zeiss).

Spatio-temporal control of mitotic cell division with one-shot of light illumination

HeLa cells on a 35 mm glass bottom dish were prepared as shown in **Counting of cells containing aligned/misaligned chromosomes using microscopic imaging**". The areas in the dish were designed as follows. Area 1; without light illumination, area 2; with single shot of 365 nm light illumination, and area 3; with single shot of 568 nm light illumination instantly after 365 nm light illumination. The non-illuminated area was covered with photomasks. After fixation, cells were visualized by the staining with DAPI.

Synthesis of 6



Scheme S1. Synthetic route of **6**. Condition: hydrazine hydrate, EtOH, 80 ºC, 8 h.

Compound **6**: Hydrazine hydrate (1:1) (242 μL, 5.00 mmol, 6.0 eq.) was added to a solution of compound **5**[S1] (416 mg, 0.83 mmol, 1.0 eq.) in EtOH (150 mL) and refluxed for 8 hours. After evaporation, the residual crude solid was purified using Isolera One to afford **6** as a yellow solid. Yield: 248 mg, 60%. ^{1}H NMR (300 MHz, CD_3OD) δ 7.86 (d, *J* = 2.1 Hz, 1H), 7.72 – 7.69 (m, 3H), 7.40 (d, *J* = 8.4 Hz, 2H), 7.12 (d, *J* = 9.0 Hz, 1H), 4.83 – 4.71 (m, 2H), 3.37 – 3.26 (m, 1H), 3.11 (dd, *J* = 13.6 ,8.8 Hz, 1H), 2.74 (s, 3H), 2.52 (br, 6H), 1.38 (d, *J* = 6.0 Hz, 6H). ESI-MS *m/z*: calcd for $[C_{25}H_{29}ClN_6O_3 + Na]^+$ 519.1882, found 519.1778.

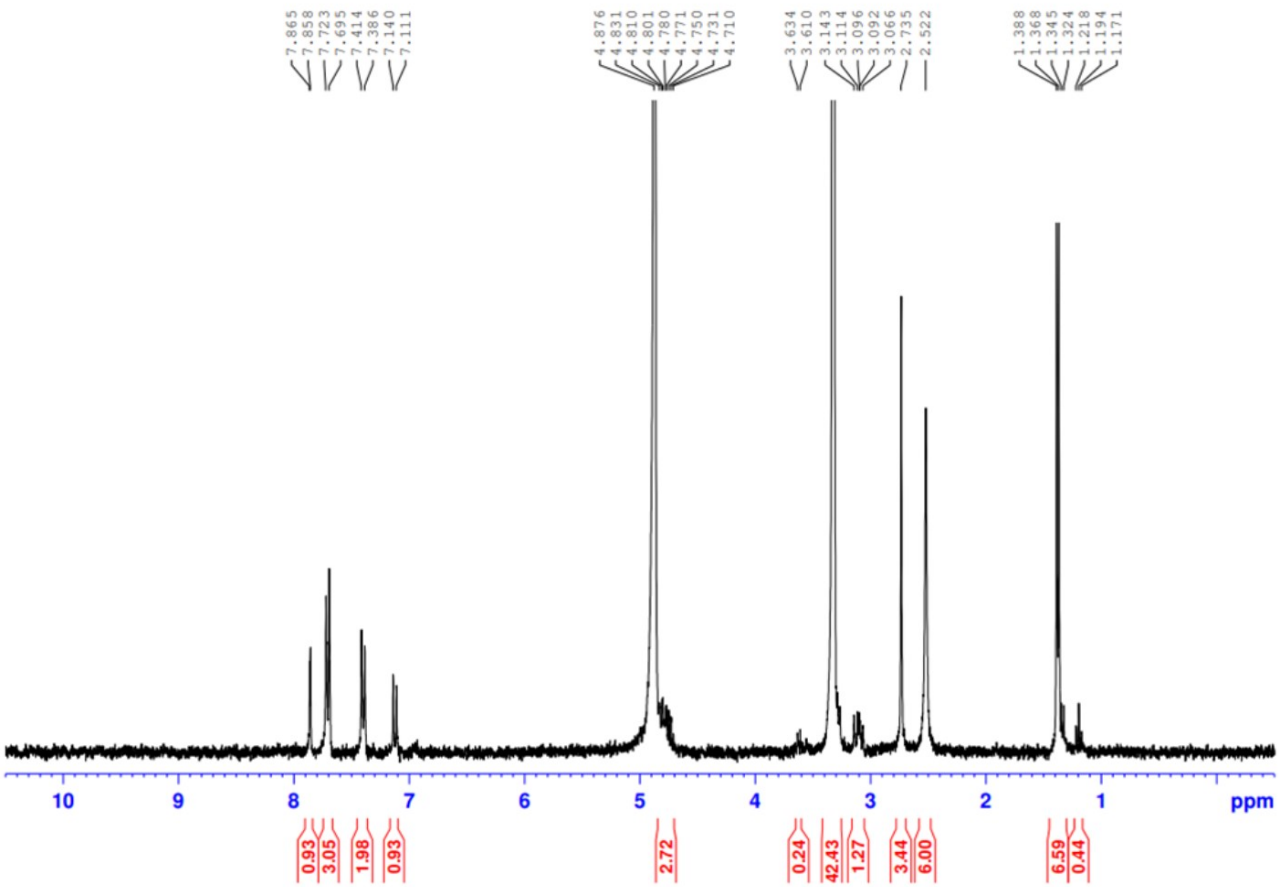
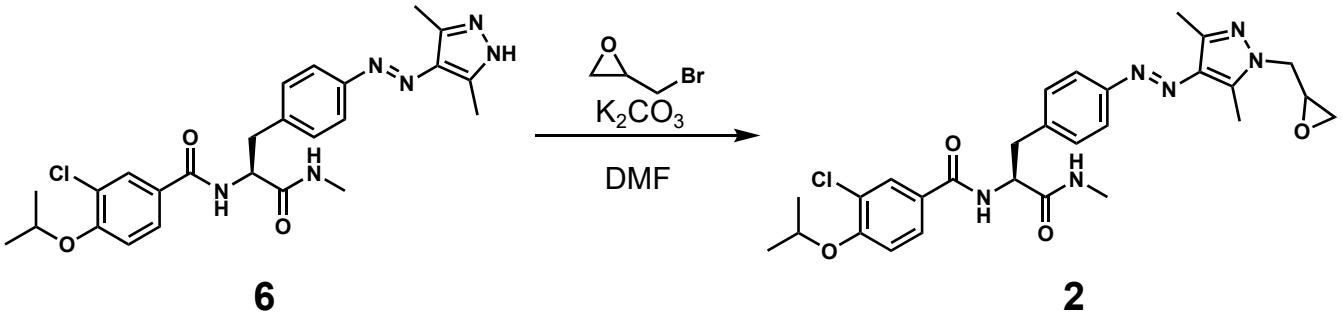


Fig. S1. ^{1}H-NMR of **5**.

Synthesis of 2



Scheme S2. Synthetic route of **2**. Conditions: epibromohydrin, K_2CO_3, DMF, r.t., 48 h.

Compound **2**: K_2CO_3 (289 mg, 0.53 mmol, 13 eq.) and epibromohydrin (125 µL, 1.53 mmol, 12 eq.) was added to a solution of **6** (62.9 mg, 0.13 mmol, 1.0 eq.) in DMF (5 mL). The resulting solution was stirred at room temperature for 48 h. It was extracted with EtOAc and sat. $NaHCO_3$aq (x 3) and washed with brine (x 1). It was dried over $MgSO_4$, filtered and evaporated *in vacuo*. The residual crude solid was purified using reverse-phase HPLC with a mobile phase gradient (acetonitrile / water with 0.1% TFA = 70 to 100% of acetonitrile in water for 30 min, 100% of acetonitrile in water for 20 min) to afford compound **2** as a yellow solid. Yield: 15.8 mg, 23%. ^{1}H NMR (300 MHz, CD_3OD) δ 7.74 (d, J = 2.1 Hz, 1H), 7.61 – 7.58 (m, 3H), 7.29 (d, J = 8.4 Hz, 2H), 7.00 (d, J = 8.7 Hz, 1H), 4.73 – 4.58 (m, 2H), 4.36 (dd, J = 15.3, 2.7 Hz, 1H), 4.04 (dd, J = 15.3, 5.4 Hz, 1H), 3.18 – 3.16 (m, 1H), 3.00 (dd, J = 13.8, 9.0 Hz, 1H), 2.73 (t, J = 4.4 Hz, 1H), 2.63 (s, 3H), 2.49 (s, 3H), 2.44 (t, J = 2.39 Hz, 1H), 2.35(s, 3H), 1.26 (d, J = 6.0 Hz, 6H). ESI-MS m/z: calcd for $[C_{28}H_{33}ClN_6O_4 + H]^+$ 553.2325, found 553.2210.

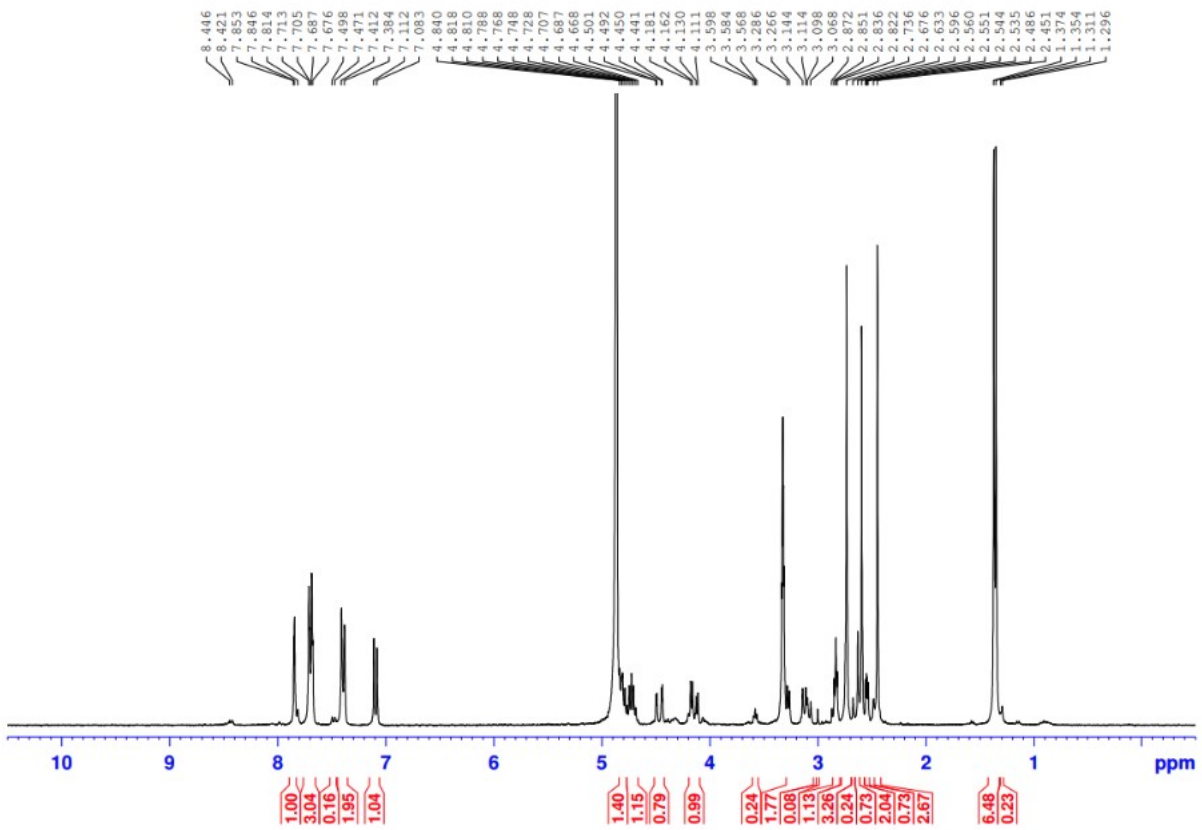
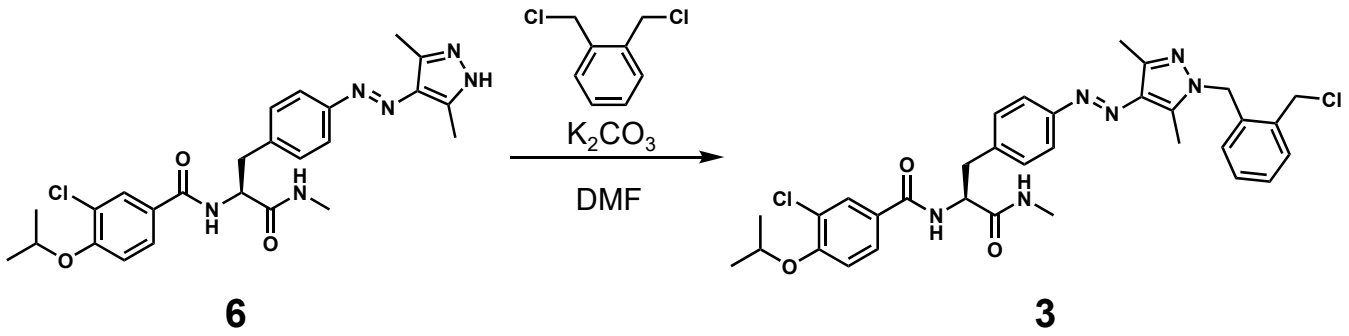


Fig. S2. ^{1}H-NMR of **2**.



Scheme S3. Synthetic route of **3**. Conditions: dichloro-*o*-xylene, K_2CO_3, DMF, r.t., 20 h.

Compound **3**: K_2CO_3 (59.0 mg, 0.13 mmol, 3.3 eq.) was added to a solution of **6** (65.1 mg, 0.13 mmol, 1.0 eq.) in DMF (30 mL). After stirring at room temperature for 10 min, dichloro-*p*-xylene (92.0 mg, 0.53 mmol, 4.0 eq.) was added to the solution. The resulting solution was stirred at room temperature for 23 h. It was extracted with EtOAc and sat. $NaHCO_3$aq (x 3) and washed with brine (x 1). It was dried over $MgSO_4$, filtered and evaporated *in vacuo*. The residual crude solid was purified using reverse-phase HPLC with a mobile phase gradient (acetonitrile / water with 0.1% TFA = 80 to 100% of acetonitrile in water for 20 min, 100% of acetonitrile in water for 40 min) to afford compound **3** as a yellow solid. Yield: 14.4 mg, 17%. ^{1}H NMR (300 MHz, CD_3OD) δ 7.75 (d, J = 2.4 Hz, 1H), 7.63 – 7.58 (m, 3H), 7.36 – 7.32 (m, 3H), 7.22 – 7.19 (m, 2H), 7.00 (d, J = 9.0 Hz, 1H), 6.59 (t, J = 2.9 Hz, 1H) 5.45 (s, 2H), 4.75 (s, 2H), 4.71 – 4.59 (m, 2H), 3.23 – 2.97 (m, 2H), 2.63 (s, 3H), 2.41 (d, J = 7.5 Hz 6H), 1.26 (d, J = 6.0 Hz, 6H). ESI-MS m/z: calcd for $[C_{33}H_{36}Cl_2N_6O_3 + H]^+$ 635.2229, found 635.1951.

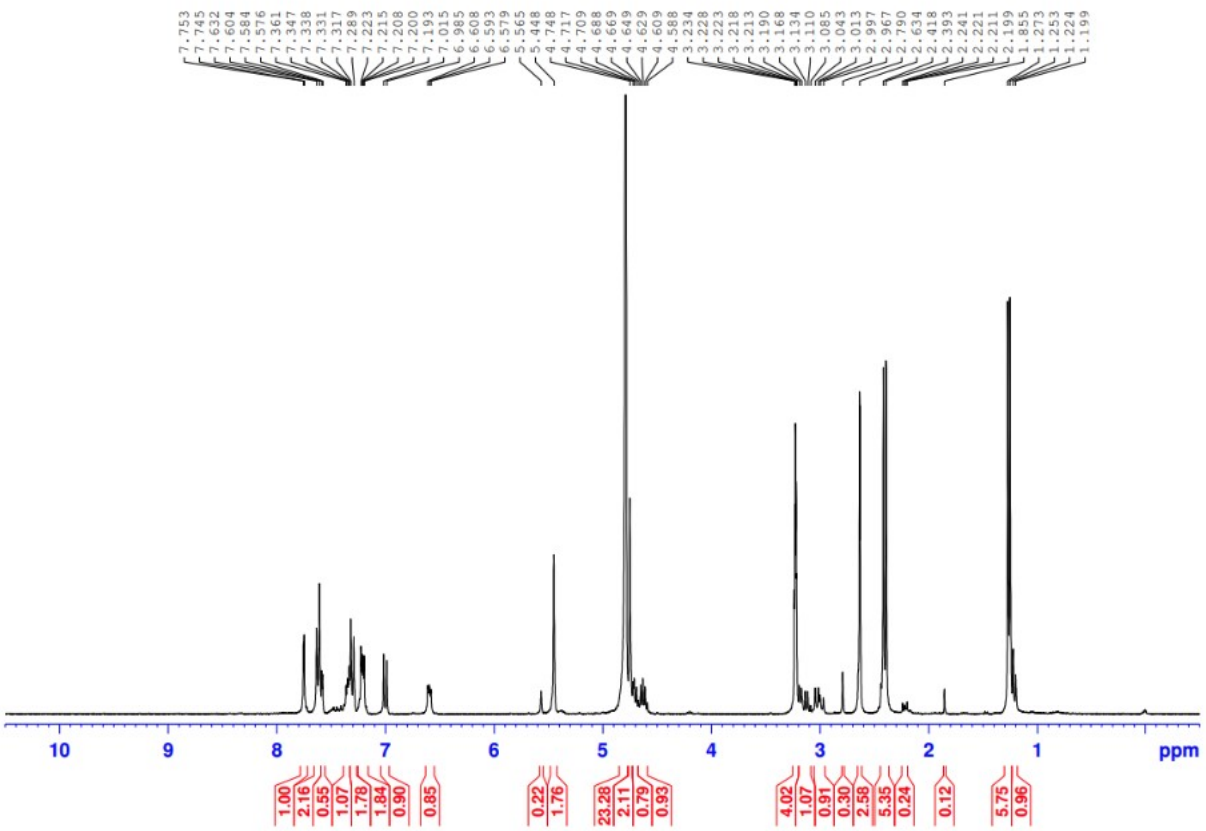
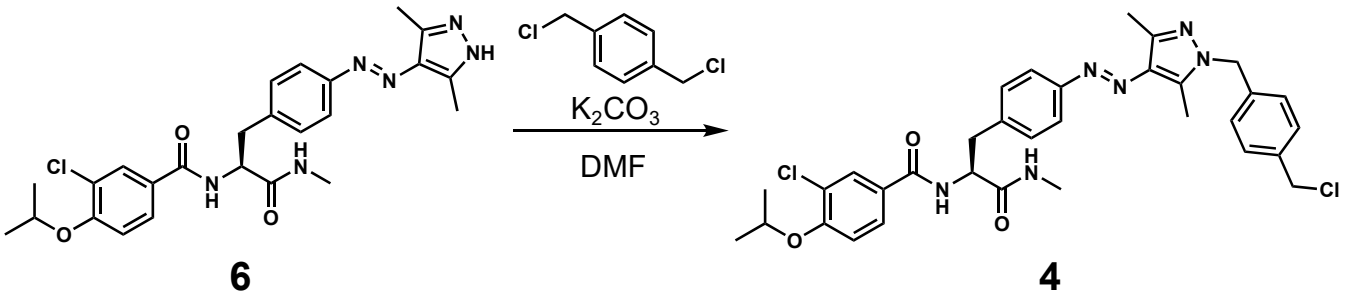


Fig. S3. ^{1}H-NMR of **3**.



Scheme S4. Synthetic route of **4**. Conditions: dichloro-*p*-xylene, K_2CO_3, DMF, r.t., 20 h.

Compound **4**: K_2CO_3 (112 mg, 0.81 mmol, 3.0 eq.) was added to a solution of **6** (133 mg, 0.27 mmol, 1.0 eq.) in DMF (50 mL). After stirring at room temperature for 10 min, dichloro-*p*-xylene (190 mg, 1.08 mmol, 4.0 eq.) was added to the solution. The resulting solution was stirred at room temperature for 20 h. It was extracted with EtOAc and sat. $NaHCO_3$aq (x 3) and washed with brine (x 1). It was dried over $MgSO_4$, filtered and evaporated *in vacuo*. The residual crude solid was purified using reverse-phase HPLC with a mobile phase gradient (acetonitrile / water with 0.1% TFA = 90 to 100% of acetonitrile in water for 10 min, 100% of acetonitrile in water for 30 min) to afford compound **4** as a yellow solid. Yield: 78.4 mg, 46%. ^{1}H NMR (300 MHz, CD_3OD) δ 7.74 (d, J = 2.1 Hz, 1H), 7.74 – 7.57 (m, 3H), 7.32 – 7.27 (m, 4H), 7.07 (d, J = 8.1 Hz, 2H), 7.00 (d, J = 8.7 Hz, 1H), 5.24 (s, 2H), 4.71 – 4.57 (m, 2H), 4.52 (s, 2H), 3.17 – 3.12 (m, 1H), 2.99 (dd, J = 13.1, 8.8 Hz, 1H), 2.62 (s, 3H), 2.45 (s, 3H), 2.37 (s, 3H), 1.26 (d, J = 6.0 Hz, 6H). ESI-MS m/z: calcd for $[C_{33}H_{36}Cl_2N_6O_3 + Na]^+$ 657.2124, found 657.2099.

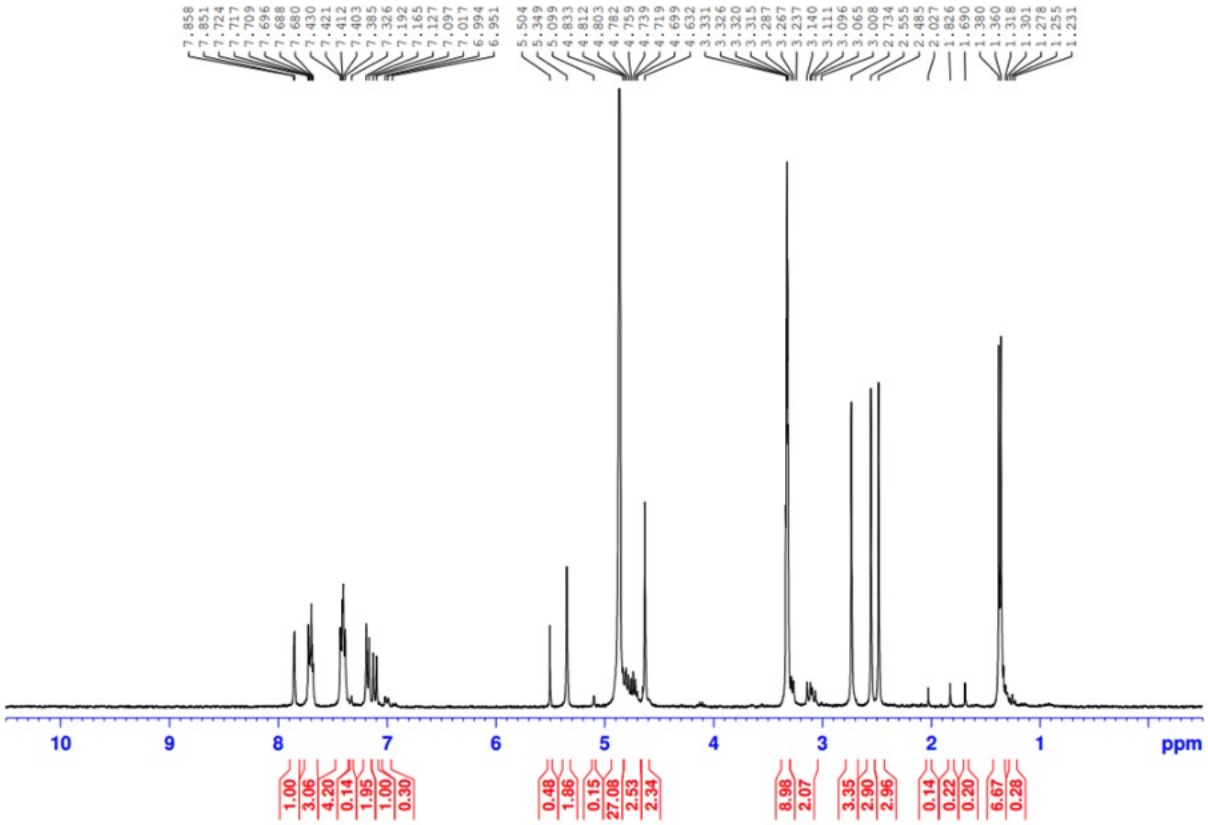


Fig. S4. ^{1}H-NMR of **4**.

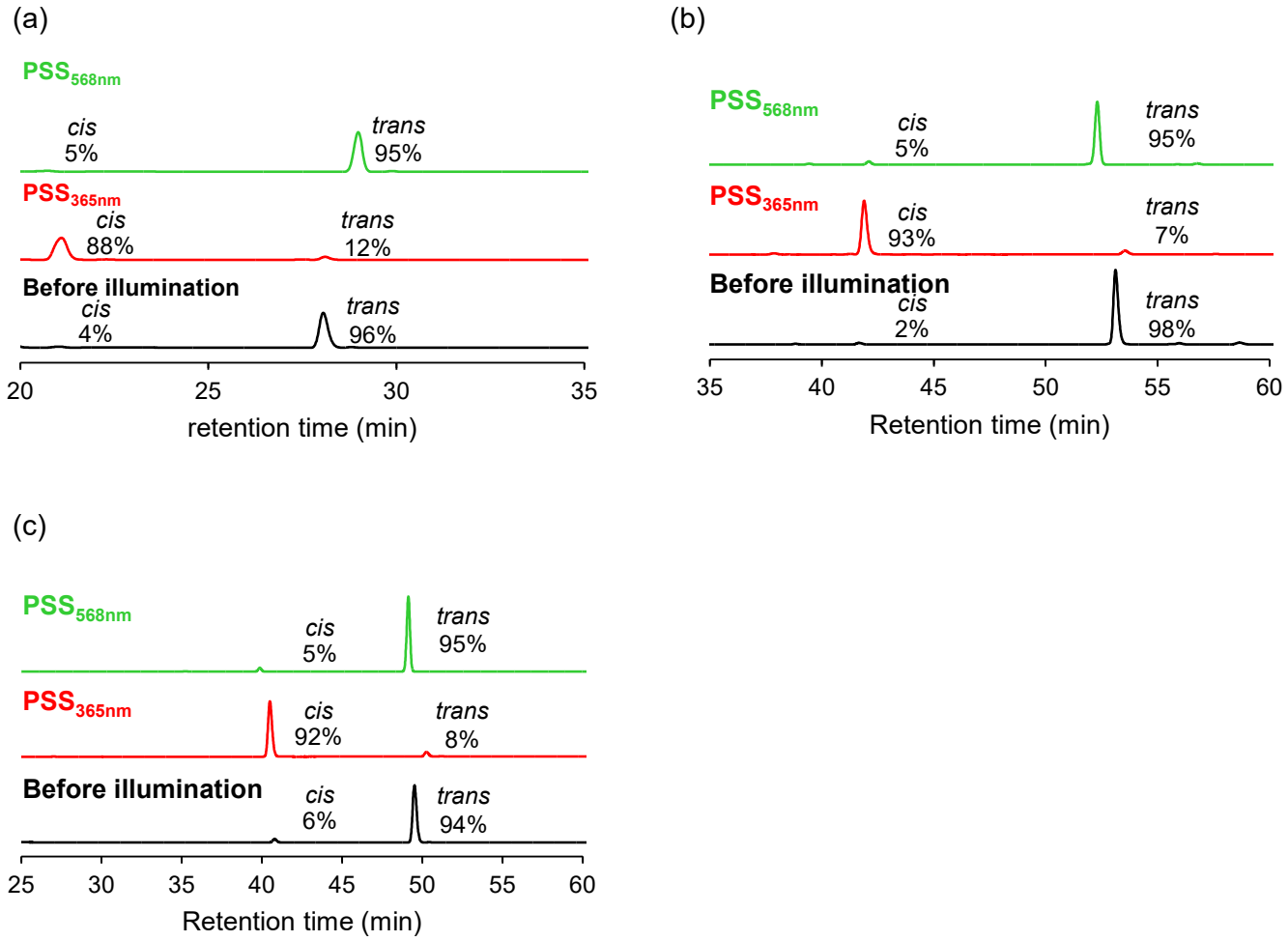


Fig. S5. HPLC analysis of the *cis/trans* ratio of **2** (a), **3** (b) and **4** (c) with each 20 μM in BRB80 buffer (pH 6.9) : acetonitrile (1:1) solution at photostationary states at 365 nm and 568 nm light illumination. Conditions used for RP-HPLC analysis; Column : 5C18-MS-II, 4.6×250 mm (Nacalai Tesque, Inc.); Eluent : CH_3CN/H_2O; Solvent gradient: 30 to 90% of acetonitrile in water for 60 min or 30 to 70% of acetonitrile in water for 40 min; Flow rate: 1 mL/min. Injection volume: 300 μL for each analysis. The *cis*/*trans* ratio was determined by the areas of the corresponding peaks at isosbestic point (294 nm).

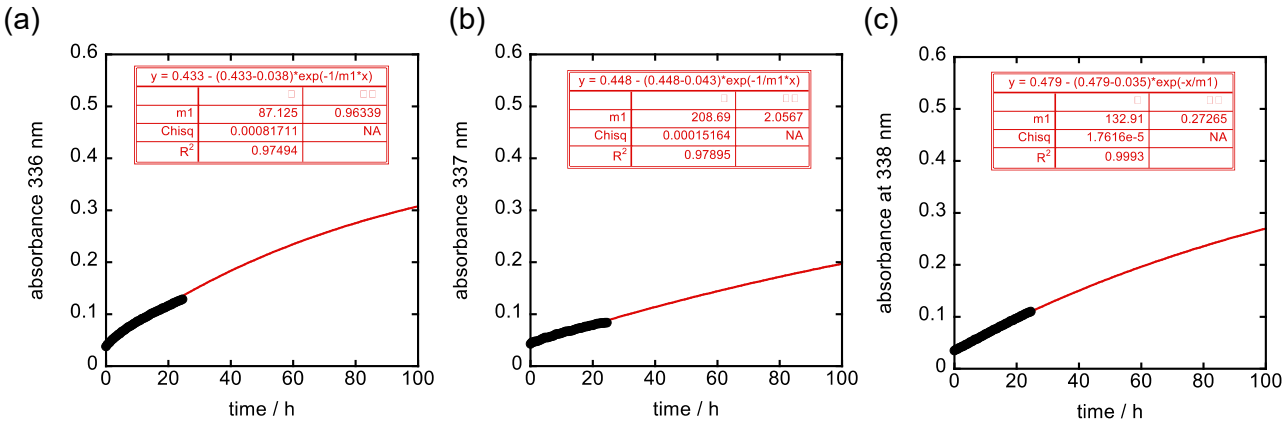


Fig. S6. Lifetime analysis of *cis* isomers of **2**-**4**. Thermal back reaction was traced by measurements of absorption changes at 336 nm (for **2**)**,** 337 nm (for **3**) and 338 nm (for **4**) with each 20 μM in BRB80 buffer (pH 6.9) : acetonitrile (1:1) solution at 37 ºC.

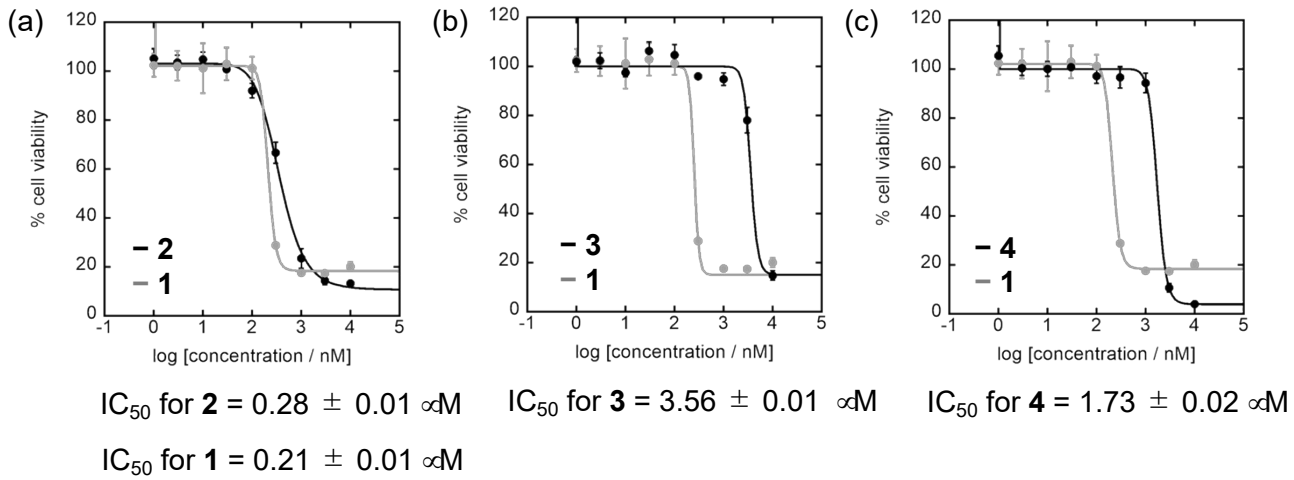


Fig. S7. Cell viability assay of **2**-**4** without light illumination vs **1**. IC_{50} values were found to be 0.28 μM for **2**, 3.56 μM for **3**, and 1.73 μM for **4** vs 0.21 μM for **1**.

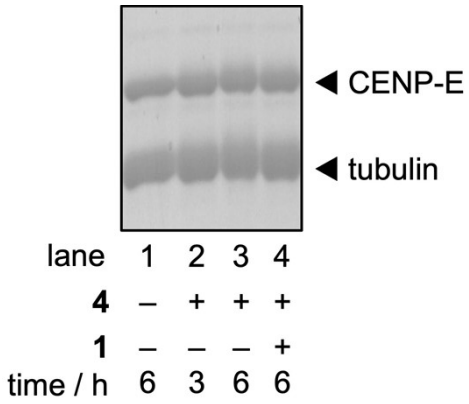


Fig. S8. The SDS-PAGE analysis of **4**-modified CENP-E. The recombinant CENP E (1 μM) and **4** (10 μM) with or without **1** (100 μM) were mixed in the presence of polymerized microtubule (5 μM) with taxol (10 μM) and ATP (1 mM) in BRB 80 buffer (pH 6.9) at 37 ºC for 6 h. The gel was stained with Coomassie Brilliant Blue. The slight smear bands at higher molecular region of CENP-E were observed in the lane 2 and 3, while the band was negligible in the presence of the competitive non-covalent inhibitor **1** (lane 4).

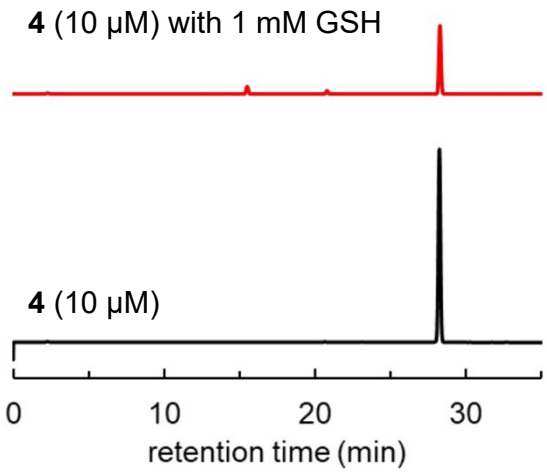


Fig. S9. The reactivity of **4** (10 μM) with 1 mM GSH for 24 h *in vitro*. HPLC condition as follows; Column : 5C18-MS-II, 4.6×250 mm (Nacalai Tesque, Inc.); Eluent : CH_3CN/H_2O; Solvent gradient : 30 to 100% of acetonitrile in water for 35 min, Flow rate : 1 mL/min. Injection volume : 300 μL. Monitored at 338 nm.

Reference

S1. N. N. Mafy, K. Matsuo, S. Hiruma, R. Uehara, N. Tamaoki, *J. Am. Chem. Soc.,* 2020, **142**, 1763.