Electronic Supplementary Information (ESI)

A visible light-controllable Rho kinase inhibitor based on a photochromic phenylazothiazole

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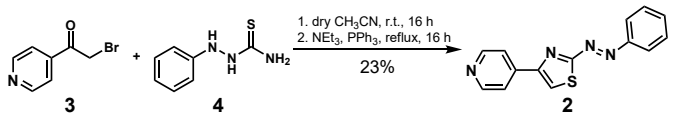
Table of Contents

1. General Methods

All reagents and solvents were purchased from commercial sources (TCI chemical, Merck, Kanto Chemical, Fujifilm Wako Pure Chemical Corporation.) and used without further purification. Thin layer chromatography was conducted on silica gel 60 F_{254}-precoated aluminum sheets (Merck) with a handy UV lamp (254 nm). Purification of the synthesized compound was performed using silica gel column chromatography system. The final compound obtained was identified by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR), and high-resolution mass spectroscopy. ^{1}H-NMR spectra were recorded on a JEOL JNM-ECX 400 spectrometer, and all chemical shifts are cited on the δ-scale in ppm relative to the signal of the solvent, and coupling constants (*J*) are reported in Hz. Proton-decoupled ^{13}C-NMR spectra were also recorded on a JEOL JNM-ECX 400 spectrometer, and all chemical shifts are reported in ppm. High-resolution mass spectra were recorded using electrospray ionization on a Thermo Scientific Exactive mass spectrometer (Thermo Fisher Scientific).

2. Experimental Methods

Synthesis of 2



Scheme S1. Synthesis of photoswitchable ROCK inhibitor 2.

The chemical synthesis of **2** was carried out following a synthetic route for the related phenylazothiazole derivatives with a slight modification[S1]. The solution of 4-(2-bromoacetyl)pyridine **3**[S2] (1.0 g, 5.0 mmol) and the corresponding thiosemicarbazide **4**[S3] (0.84 g, 5.0 mmol) in dry acetonitrile (100 mL) was stirred for 16 h at room temperature. Triethylamine (0.70 ml, 5.0 mmol) and triphenylphosphine (1.32 g, 5.0 mmol) were added and refluxed for 16 h. The reaction mixture was extracted with AcOEt and sat. $NaHCO_3$aq. The organic phase was dried over $MgSO_4$, and filtered. The resulting solution was dried *in vacuo*. The crude residue was purified by silica gel column chromatography (EtOAc:Hexane (7:3), isocratic) to afford compound **2** (0.30 g, 23%) as a dark orange solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.73 (dd, J = 4.4, 1.6 Hz, 2H), 8.09-8.06 (2H, m), 7.90 (dd, J = 4.6, 1.8 Hz, 2H), 7.84 (s, 1H), 7.62-7.55 (3H, m). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 177.23, 153.46, 151.51, 150.68, 140.84, 133.56, 129.55, 124.28, 120.49, 118.56. HR-MS (ESI) for $[M + H]^+$ calcd. 267.0699, found 267.0702.

Evaluation of the photophysical properties of ROCK inhibitor 2

UV-Vis absorption spectra were recorded using a Shimadzu UV-2600 spectrophotometer. The *cis-trans* photoisomerization of **2** (20 μM) in an aqueous solution (acetonitrile:BRB80 buffer = 1:1) was induced by light illumination with 405 and 525 nm light-emitting diodes (CL-1503 with CL-H1-405-9-1 and CL-H1-525-7-1, ASAHI SPECTRA). BRB80 buffer : 80 mM PIPES (pH 6.9), 1 mM $MgCl_2$, and 1 mM EGTA. The time duration to reach the photostationary states was optimized by the absorbance measurements with the light illumination at 405 nm (100 mW/cm^2) and 525 nm (100 mW/cm^2) to samples for every 10 sec. These experiments revealed that the light illumination at 405 nm for 20 sec and at 525 nm for 30 sec was enough to reach the photostationary states.

***In vitro* kinase assay**

ROCK-based kinase assay was carried out using ADP-Glo kinase assay (Promega). Five nanomolar concentration of ROCK1 (amino acid residue: 17–535, recombinant, SignalChem) was incubated with DMSO (control, 0.5%) or inhibitors in a aqueous solution containing 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 0.1 mM DTT, 0.01% Brij-35, and 0.1 mg/mL BSA. Kinase reactions in 384 well

plates were initiated by the addition of the substrate of 5 μM ATP at 25 °C. After 60 min of incubation, ADP-Glo system was used for the detection of consumption of ATP. The chemiluminescent signals in each well were detected using a plate reader (Infinite 200 Pro M Plex, Tecan). The half maximal inhibitory concentration (IC_{50}) was calculated by a nonlinear regression analysis (four-parameter logistic model), where log[inhibitor] was plotted against the percentage of ROCK activity. In this assay, the samples were illuminated with the light of 405 nm (100 mW/cm^2, for 20 sec) and 525 nm (50 mW/cm^2, for 30 sec) for every 5 min for the induction of the *cis-trans* photoisomerization of **2**.

Cell culture

Balb3T3 (mouse fibroblast) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing L-glutamine and phenol red (Fujifilm Wako Pure Chemical Corporation), supplemented with 5% fetal bovine serum (FBS; Corning), and 1% antibiotic and antimycotic solution (Merck), at 37 °C in the presence of 5% CO_2 in a humidified atmosphere.

Western blotting of MLC-2 and phosphorylated MLC-2

Balb3T3 cells (2×10^5 cells) were seeded in DMEM with 5% FBS in a 35 mm culture dish. Cells were

starved overnight by the incubation in DMEM with 0.1% FBS. DMSO or inhibitors (100 μM) were added to cells in DMEM with 10% FBS, and cells were incubated for 60 min with or without light illumination for every 5 min to maintain the photostationary states (405 nm : 10 mW/cm^2 for 5 sec, 525 nm : 50 mW/cm^2 for 5 sec). The cells were rinsed three times with D-PBS (Dulbecco's phosphate-buffered saline) and RIPA buffer (150 μL/dish) was added to lyse cells on ice. The cell lysates were collected and centrifuged at 16,000 g for 20 min at 4 ºC. The supernatant was mixed with the sample buffer (4x) and boiled for 3 min. These samples were subjected to SDS-PAGE with 4-20% gradient gels (Bio-Rad). Western blotting was performed using antibodies against phosphorylated MLC-2 (Phospho-Myosin Light Chain 2 (Ser19) Antibody, 3671, Cell Signaling Technology), MLC-2 (Myosin Light Chain 2 Antibody, 3672, Cell Signaling Technology) and GAPDH (anti-GAPDH antibody, GTX100118, GeneTex, as the loading control).

Fluorescence imaging of actin stress fibers in cells

Balb3T3 cells (1×10^5 cells) were seeded in DMEM with 5% FBS on a 35 mm glass bottom dish (IWAKI). After incubation overnight, the cells were starved overnight by the incubation in DMEM with 0.1% FBS. DMSO or inhibitors (100 μM) in DMEM with 10% FBS, and cells were incubated for 60 min with or

without light illumination every 5 min to maintain the photostationary states (405 nm : 40 mW/cm^2 for 5 sec, 525 nm : 50 mW/cm^2 for 5 sec). The cells were rinsed three times with D-PBS. Cells were fixed with 4% paraformaldehyde in PBS (Fujifilm Wako Pure Chemical Corporation) for 30 min at room temperature. After washing three times with D-PBS with 100 mM glycine, 0.1% Triton X-100 in D-PBS was added for 5 min at room temperature to enhance the membrane permeability. After washing with D-PBS, the cells were stained with 10 nM rhodamine-phalloidin (Fujifilm Wako Pure Chemical Corporation) and 1.0 μg/mL 4',6-diamidino-2-phenylindole (DAPI, Fujifilm Wako Pure Chemical Corporation) in PBS with 1% BSA and incubated at 4ºC overnight. The fluorescence images were captured using a confocal laser scanning microscopy system (Nikon A1 and Ti-E, Nikon) equipped with a Plan Apo VC ×60 objective lens (NA 1.40, Nikon), and 405 and 561 nm lasers for DAPI and rhodamine-phalloidin excitation, respectively. Images were acquired and analyzed with a NIS Elements software (Nikon).

3. Results

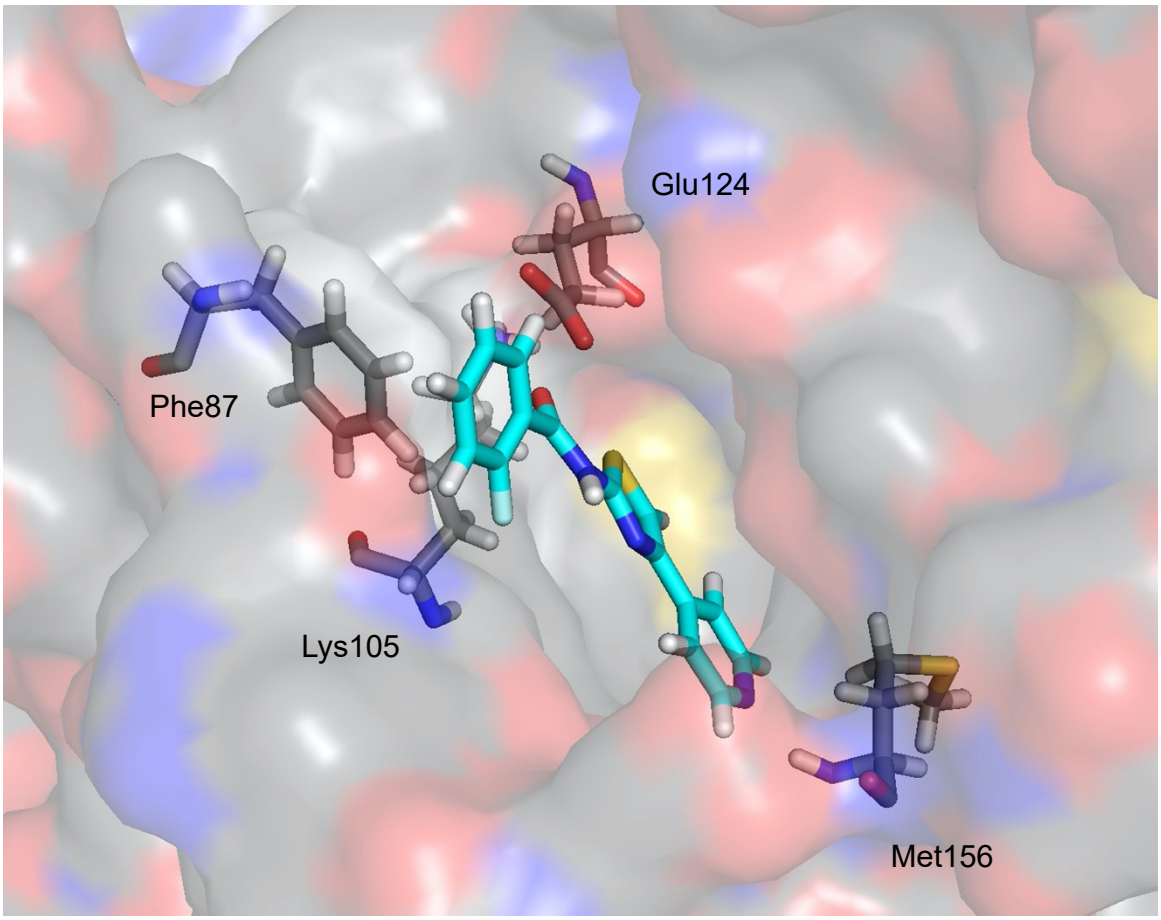


Figure S1. X-ray crystal structure of **1**, which was shown with cyan-colored carbon, bound to ROCK1 (PDB : 4YVC)[S4].

The pyridine ring in **1** is buried in the hydrophobic pocket composed of Tyr155, Met156, Leu205, and Phe368. Especially, the NH backbone of Met156 was interacted with the pyridine ring. The carbonyl group in the amide of **1** interacts with the side chain of Lys105 through a hydrogen bond. The 2-fluorophenyl ring might be interaction with the hydrophobic surface of ROCK1 composed of Phe87.

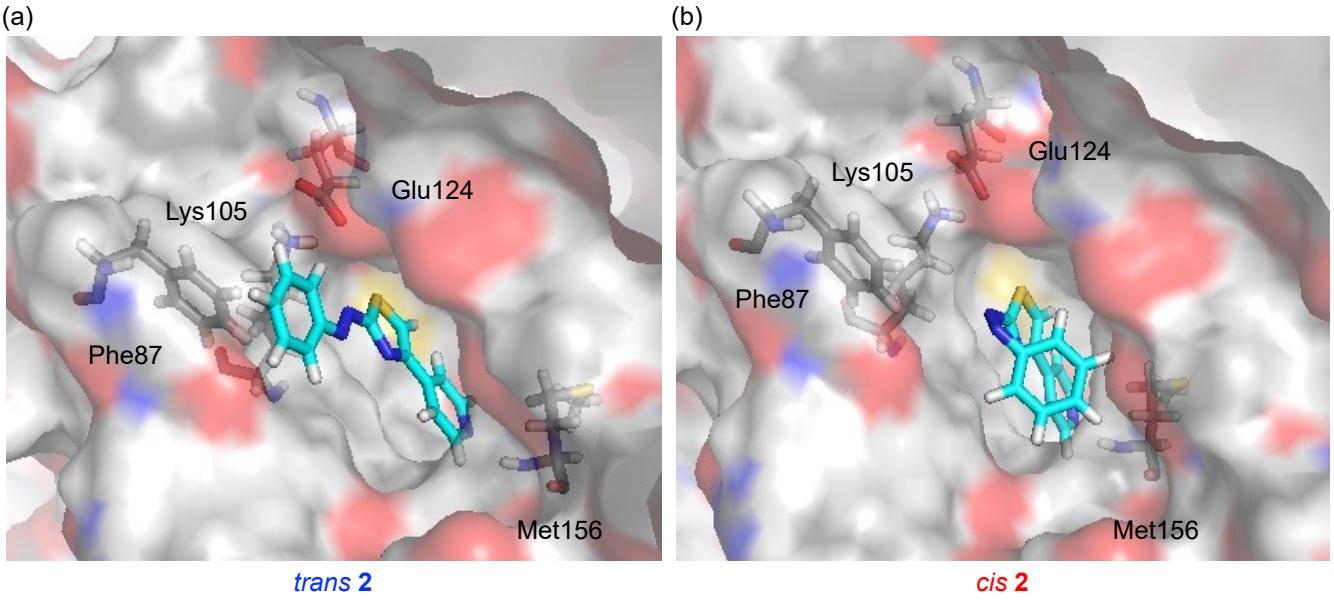


Figure S2. Docking simulation results of (a) *trans* **2** and (b) *cis* **2** with ROCK1 (PDB : 4YVC). Both isomers of **2** were shown with cyan-colored carbon.

Through the simulation using a DiscoveryStudio software, the docking scores (LibDockScore) were found to be 80.7751 for *trans* **2** and 56.7335 for *cis* **2**. High score indicates the high stability of the simulated complex. The pyridine group in both isomers showed the hydrogen bonding to the backbone NH of Met156. The phenyl group in *trans* **2** was interacted with the hydrophobic surface composed of Phe87 as shown in the cased of **1** (Figure S1). On the other hand, that in *cis* **2** was faced to the solvent because of the bent structure of the *cis* isomer, which indicated the less interaction with ROCK1 than *trans* **2**.

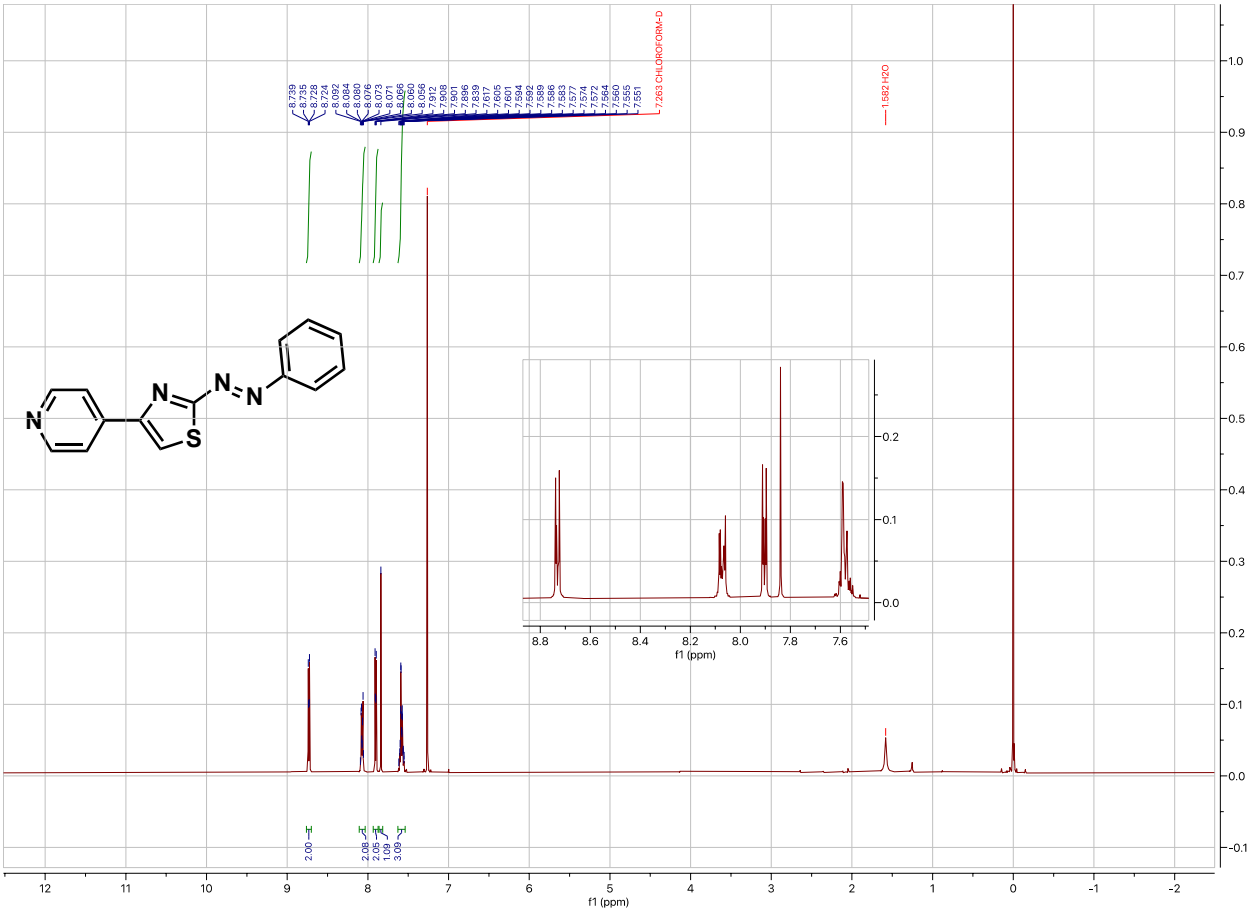


Figure S3. ^{1}H-NMR of **2**.

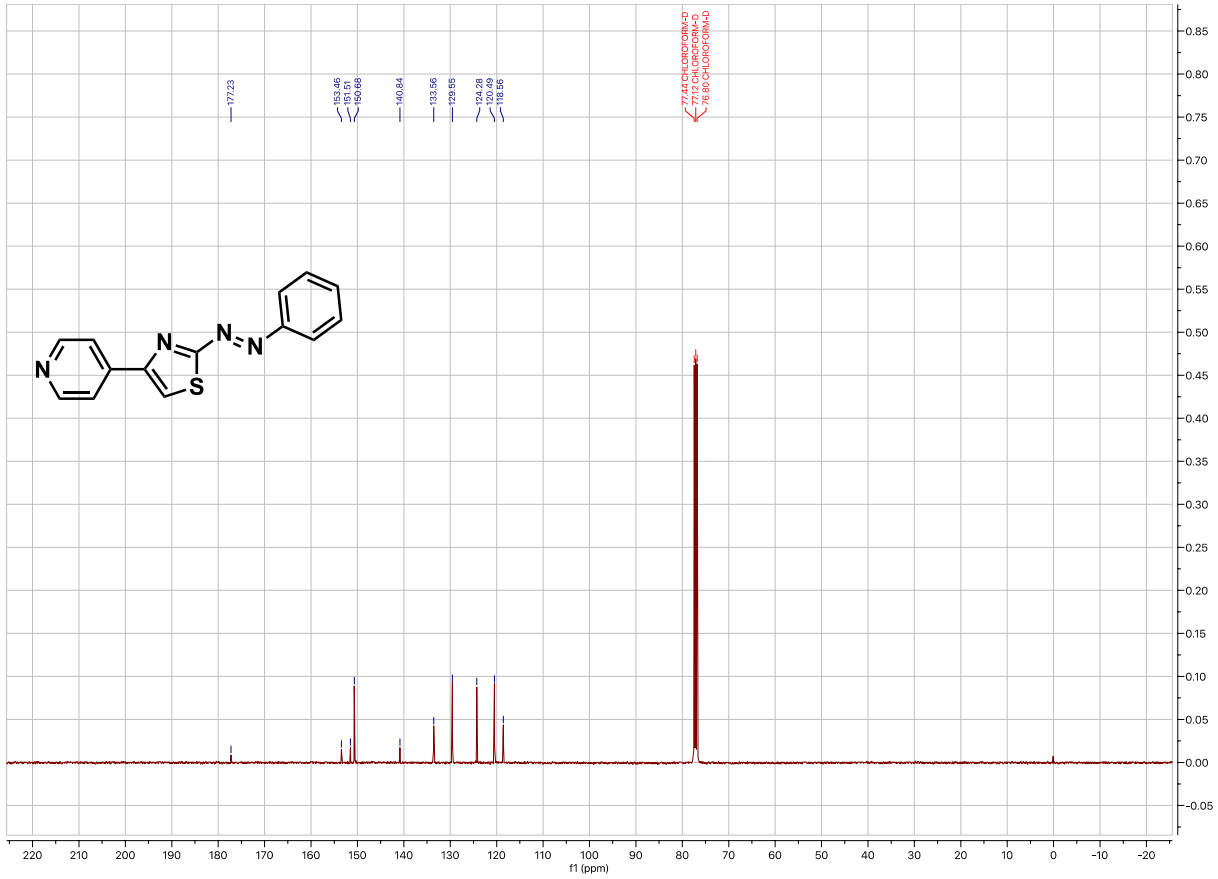


Figure S4. ^{13}C-NMR of **2**.

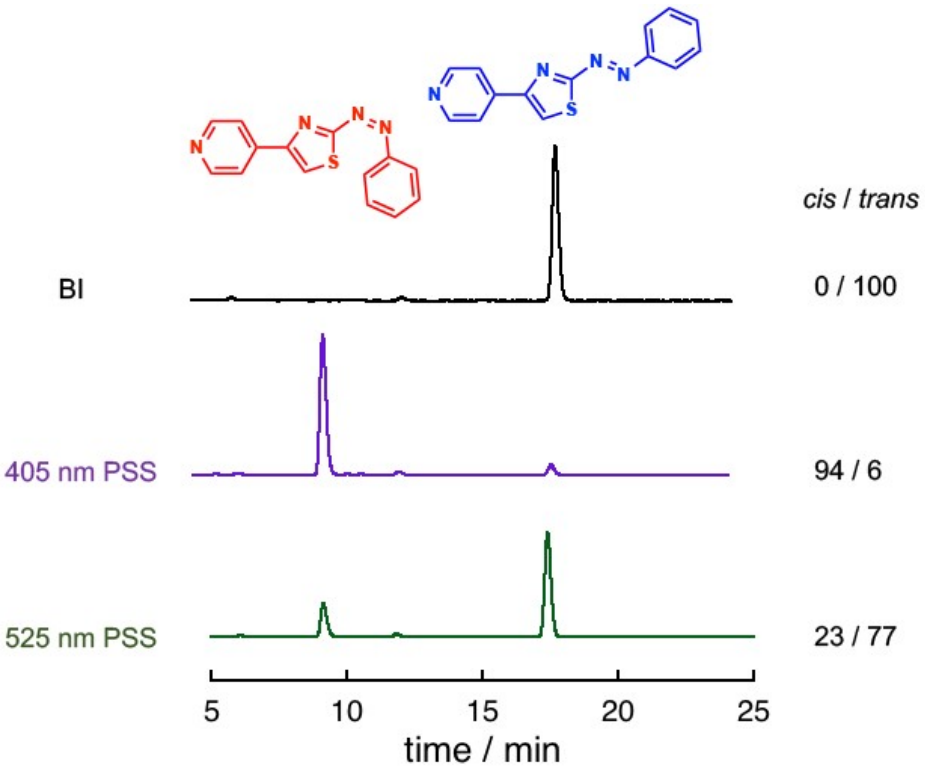


Figure S5. High performance liquid chromatography (HPLC) analysis of *cis/trans* isomeric proportions of **2** before light illumination (BI) and at photostationary states (PSS) with 405 nm and 525 nm light illumination. Compound **2** (20 μM) in aqueous solution (acetonitrile : BRB80 buffer = 1 : 1) was prepared with or without light illumination at 405 nm (100 mW/cm^2, for 20 sec) and 525 nm (100 mW/cm^2, for 30 sec) at 25 ºC. The samples were subjected to HPLC analysis. HPLC conditions : CH_3CN/H_2O = 50/50–80/20 for 30 min. Detection at 268 nm of an isosbestic point of **2**.

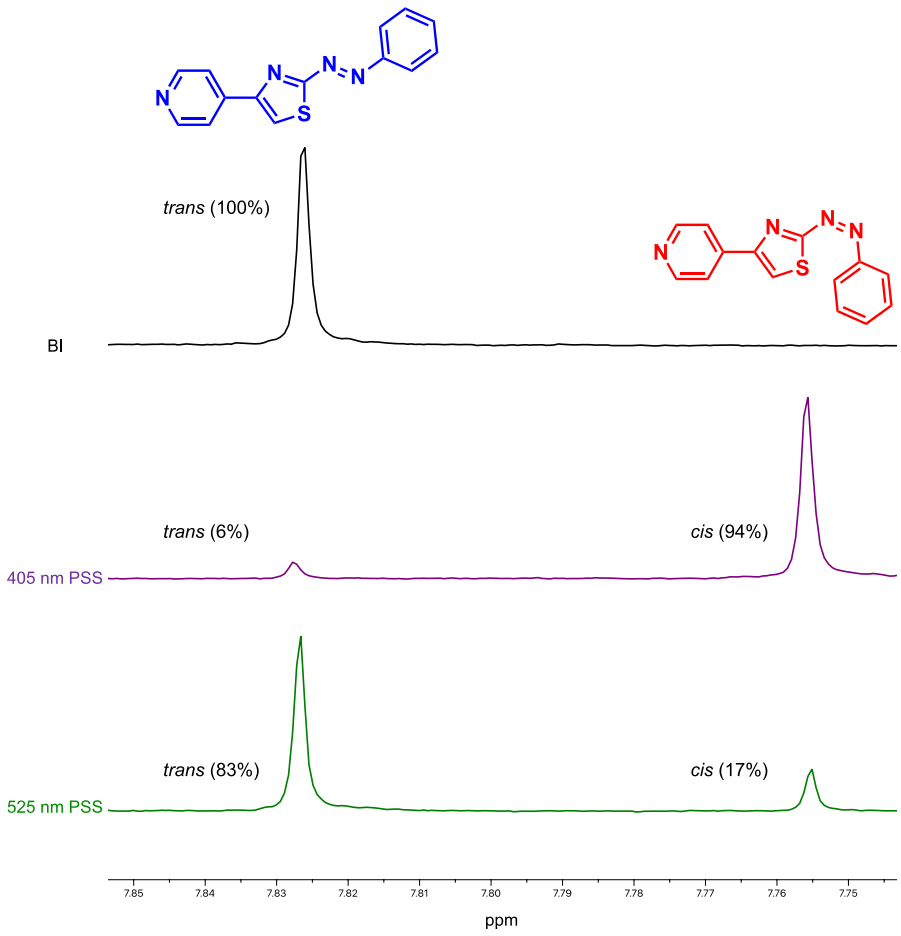


Figure S6. NMR analysis of *cis/trans* isomeric proportions of **2** before light illumination (BI) and at photostationary states (PSS) with 405 nm and 525 nm light illumination. Compound **2** in $CDCl_3$ was illuminated with 405 nm (100 mW/cm^2, for 25 min) and 525 nm (100 mW/cm^2, for 25 min) at room temperature to reach the photostationary states before NMR measurements.

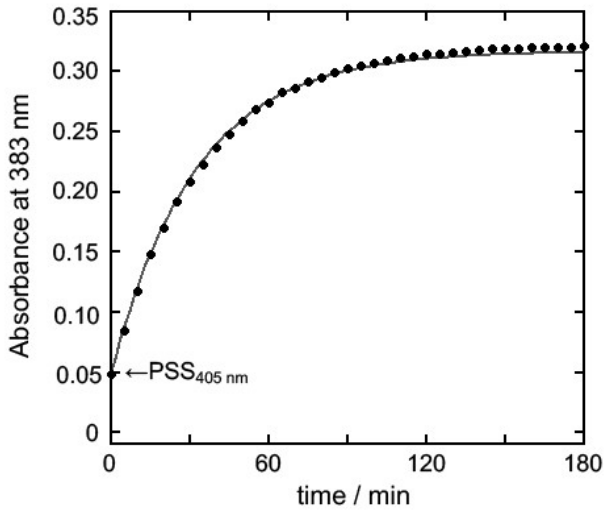


Figure S7. Thermal stability of *cis*-**2** (20 μM) in an aqueous solution (acetonitrile : BRB80 = 1 : 1) at 37 ºC. The lifetime of *cis*-**2** is calculated as 32 min. The fitting equation is $Abs_{BI} - (Abs_{BI} - Abs_{PSS405\ nm}) \times \exp(-t/\tau)$, where Abs_{BI} is the absorbance before light illumination, $Abs_{PSS405\ nm}$ is the absorbance at photostationary state under 405 nm light illumination, τ is the lifetime of *cis*-**2**, and t is time (min).

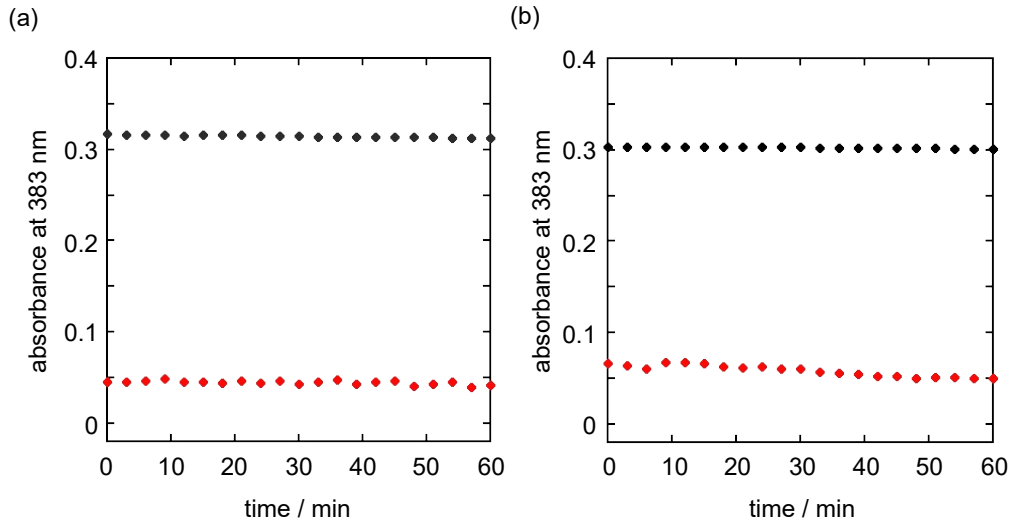


Figure S8. Reductant stability of **2** (20 μM) in aqueous buffer (acetonitrile : BRB80 buffer = 1 : 1) towards (a) 0.1 mM DTT and (b) 1.0 mM GSH for 1 h with and without light illumination at 405 nm at 25 ºC. Black circle : without light illumination. Red circle : with light illumination at 405 nm (100 mW/cm^2, 10 sec) just before every absorbance measurement.

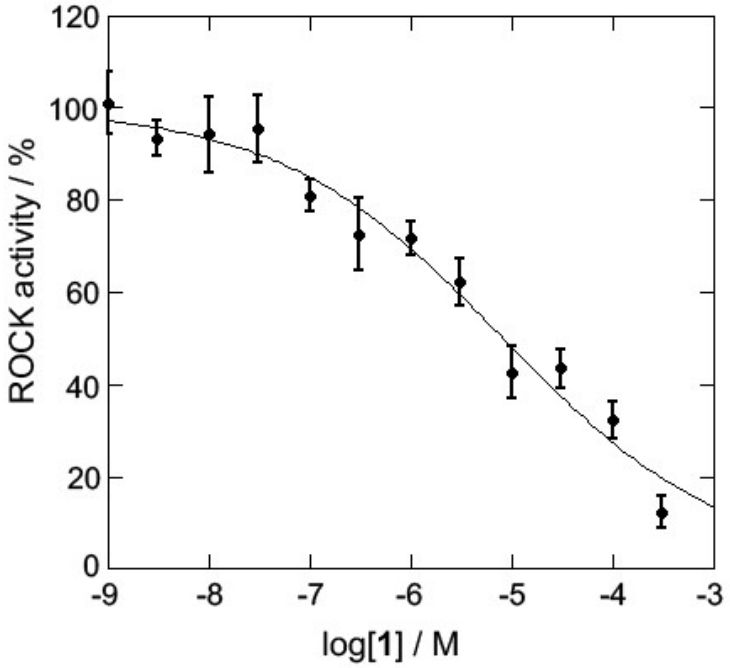


Figure S9. *In vitro* kinase assay of non-photoresponsive ROCK inhibitor **1** (IC_{50} = 8.2 ± 1.8 μM).

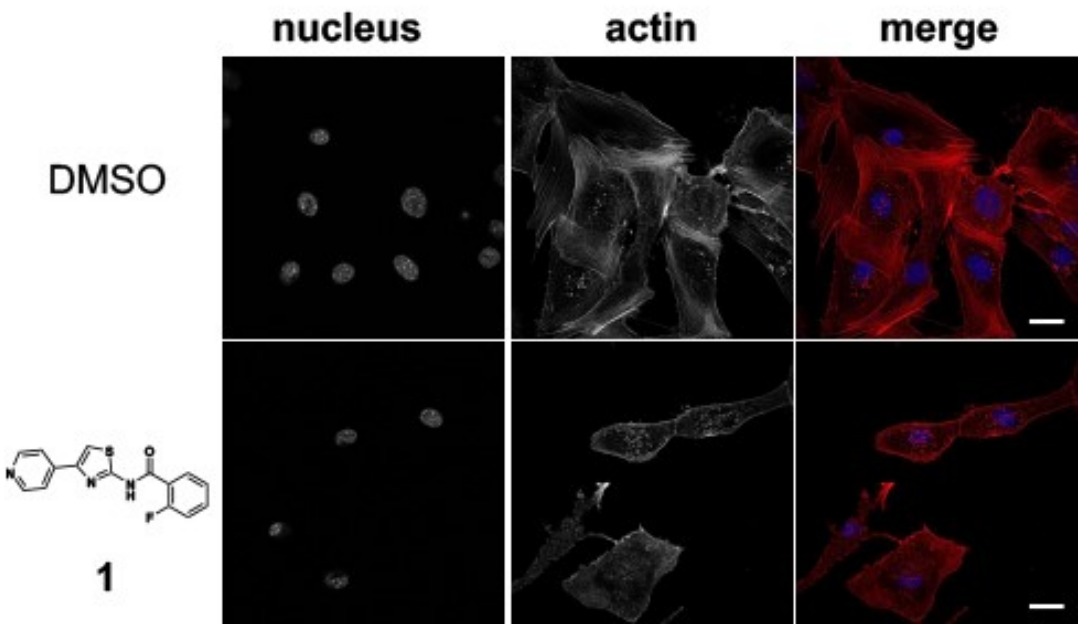


Figure S10. Fluorescence imaging of actin stress fibers in the presence of DMSO (0.5 %) or ROCK inhibitor **1** (100 μM) without light illumination. Scale bar : 25 μm.

4. Acknowledgement

We are grateful to the Instrumental Analysis Division of the Global Facility Center (Creative Research Institution, Hokkaido University) for HR-MS measurements with Thermo Scientific Exactive mass spectrometer (Thermo Fisher Scientific) and to the Nikon Imaging Center (Hokkaido University) for being very helpful with confocal microscopy, image acquisition, and analysis.

5. References

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