Electronic Supplementary Information (ESI)

Design of spontaneously blinking fluorophores for live-cell super-resolution imaging based on quantum-chemical calculations

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$$\begin{cases} -\dfrac{d[open]}{dt} = k_{close}[open] - k_{open}[close] \\ 0 = k_{close}[open]_e - k_{open}[close]_e \\ [open] + [close] = [open]_e + [close]_e \end{cases}$$

[ ]$_e$: concentration at equilibrium

$$-\frac{d([open] - [open]_e)}{dt} = (k_{close}+k_{open})([open] - [open]_e)$$

$$\tau_{cycl} = \frac{1}{k_{close} + k_{open}} \sim \frac{1}{k_{close}}$$

(under pH condition where closed form prevails)

Fig. S1 Lifetime (τ_{cycl}) of open form in spirocyclization equilibrium.

$$\begin{cases} pH = pK_{aOH} + \log\dfrac{[O_B]}{[O_A]} \quad \leftrightarrow \quad [O_B] = 10^{pH-pKaOH}[O_A] \\ V_{close} = k_A[O_A] + k_B[O_B] \\ [HMR \cdot open] = [O_A] + [O_B] \sim [O_A] \end{cases}$$

※ **pH << pK_{aOH}**

$$V_{close} = (k_A + k_B 10^{pH-pKaOH})[HMR \cdot open]$$

$$k_{close} = k_A + k_B 10^{pH-pKaOH}$$

$$\tau_{cycl} = \frac{1}{k_{close}+k_{open}} \sim \frac{1}{k_{close}} = \frac{1}{k_A+k_B 10^{pH-pKaOH}} = \frac{1}{k_A} \cdot \frac{1}{1+\frac{k_B}{k_A}10^{pH-pKaOH}}$$

Fig. S2 Calculation of τ_{cycl} based on the competition between the two elemental reactions as a function of pH.

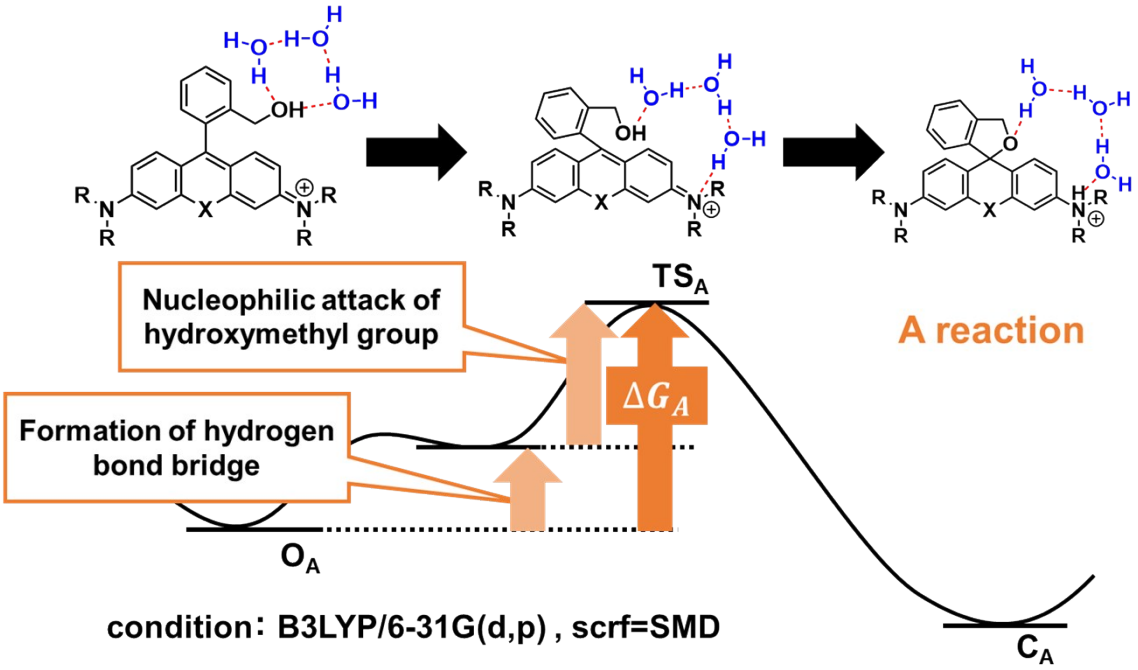


Fig. S3 Composition of the activation energy in the A reaction.

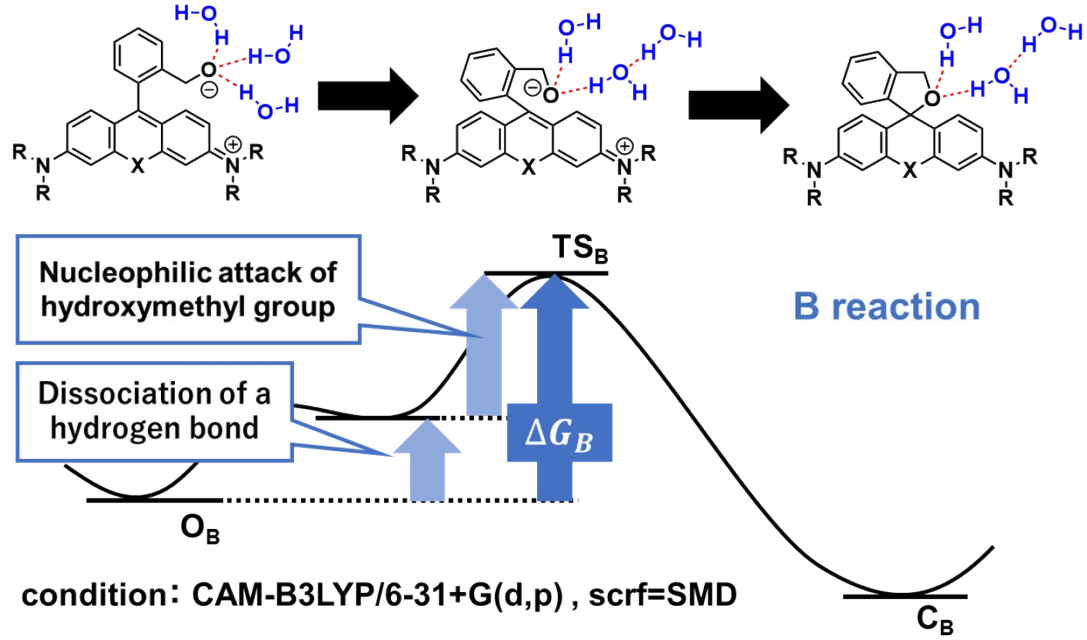
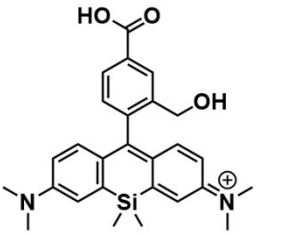
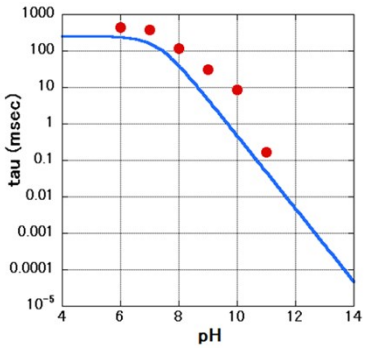


Fig. S4 Composition of the activation energy in the B reaction.



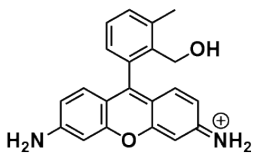
4-COOH-HMSiR

pK_{cycl} = 5.8 (measured)



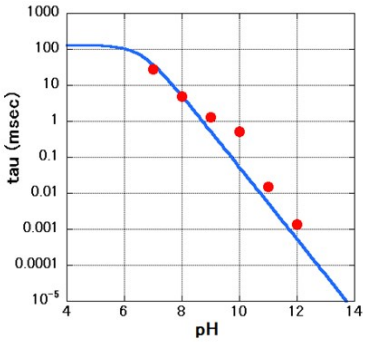
pH	measured τ_{cycl} (ms)	calculated τ_{cycl} (ms)
6	440	240
7	380	170
8	120	40
9	31	4.7
10	8.6	0.47
11	0.16	0.047

Fig. S5 Comparison of measured and calculated τ_{cycl} (lifetimes of the open form: the duration until the open form reverts to the closed form) of 4-COOH-HMSiR. Transient absorption by laser flash photolysis (LFP) measurements were performed with 4-COOH-HMSiR (50 μM) in 10 mM sodium phosphate buffer (excitation: 308 nm). See also Table S1 for τ_{cycl} calculation.



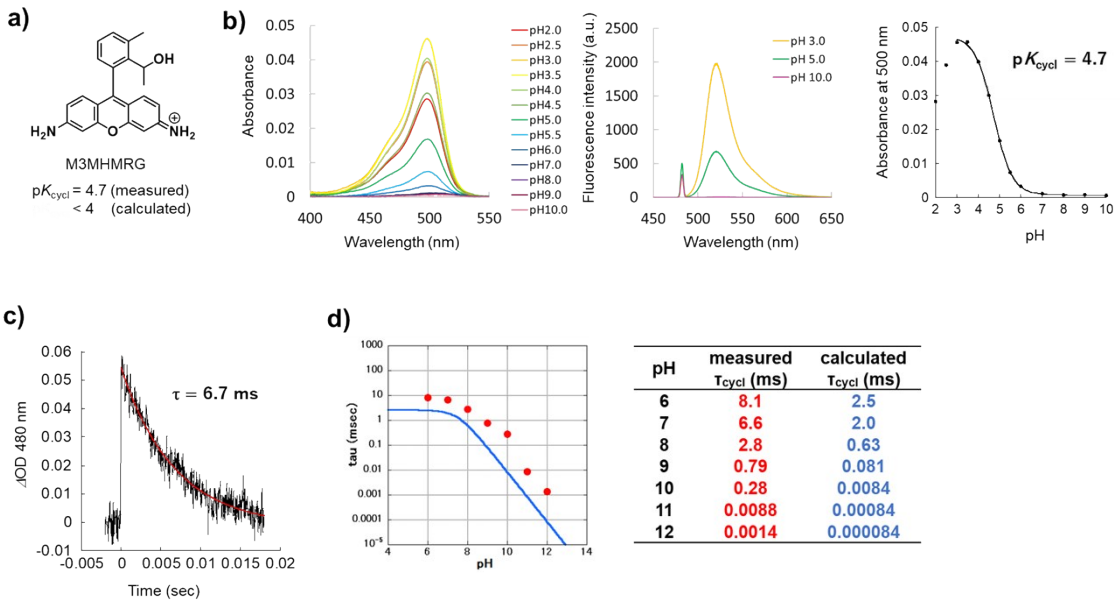
3MHMRG

pK_{cycl} = 6.6 (measured)
= 6.1 (calculated)



pH	measured τ_{cycl} (ms)	calculated τ_{cycl} (ms)
7	27	38
8	4.9	5.1
9	1.3	0.53
10	0.51	0.053
11	0.015	0.0053
12	0.0014	0.00053

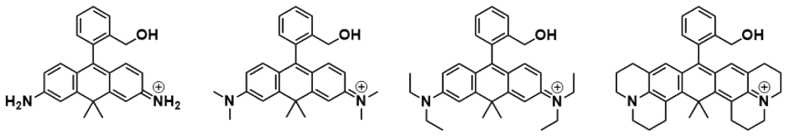
Fig. S6 Comparison of measured and calculated τ_{cycl} (lifetimes of the open form: the duration until the open form reverts to the closed form) of 3MHMRG. Transient absorption measurements were performed with 3MHMRG (50 μM) in 10 mM sodium phosphate buffer (excitation: 266 nm). See also Table S1 for τ_{cycl} calculation.



pH	measured τ_{cycl} (ms)	calculated τ_{cycl} (ms)
6	8.1	2.5
7	6.6	2.0
8	2.8	0.63
9	0.79	0.081
10	0.28	0.0084
11	0.0088	0.00084
12	0.0014	0.000084

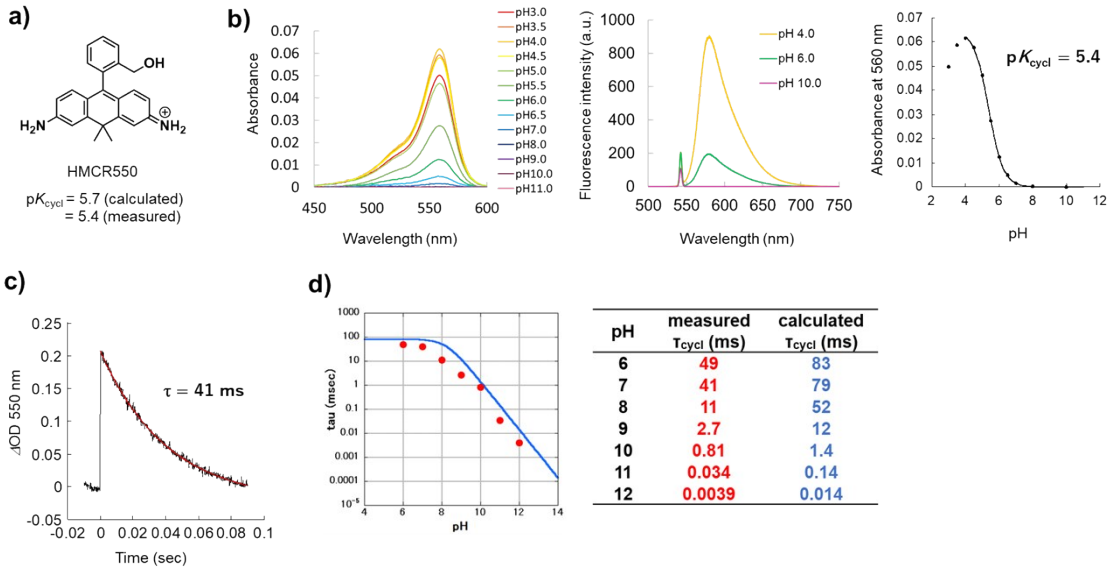
Fig. S7

a) Chemical structure of M3MHMRG. b) Absorption/fluorescence spectra of 1 µM M3MHMRG and pH dependency of absorbance at 500 nm in 100 mM sodium phosphate buffer containing 0.1% DMSO as cosolvent. c) Representative transient absorption decay measured at pH 7.0. d) Comparison of measured and calculated τ_{cycl} (lifetimes of the open form: the duration until the open form reverts to the closed form) of M3MHMRG. Transient absorption measurements were performed with M3MHMRG (50 µM) in 10 mM sodium phosphate buffer (excitation: 266 nm). See also Table S1 for τ_{cycl} calculation.



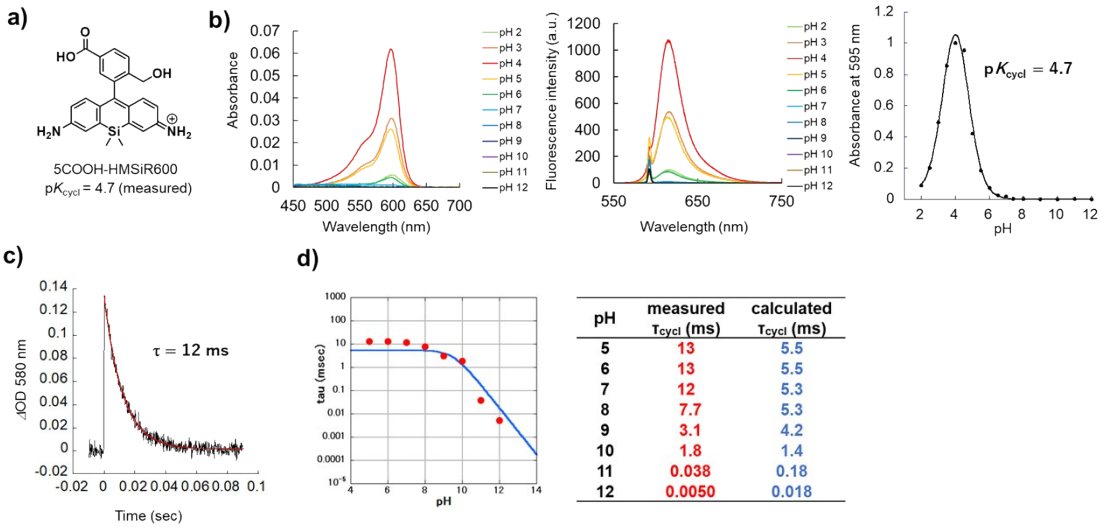
	HMCR550	HMTMCR	HMCRB	HMCR101
Calculated pK_{cycl}	5.7	7.5	8.6	10.7
Calculated τ_{cycl} (pH 7.4)	72.5 ms	718 ms	3.6 s	3090 s

Fig. S8 Calculation of pK_{cycl} and τ_{cycl} values of candidate fluorophores based on C-rhodamine. See also Table S1 for τ_{cycl} calculation.



pH	measured τ_{cycl} (ms)	calculated τ_{cycl} (ms)
6	49	83
7	41	79
8	11	52
9	2.7	12
10	0.81	1.4
11	0.034	0.14
12	0.0039	0.014

Fig. S9 a) Chemical structure of HMCR550. b) Absorption/fluorescence spectra of 1 µM HMCR550 and pH dependency of absorbance at 560 nm in 100 mM sodium phosphate buffer containing 0.1% DMSO as cosolvent. c) Representative transient absorption decay measured at pH 7.0. d) Comparison of measured and calculated τ_{cycl} (lifetimes of the open form: the duration until the open form reverts to the closed form) of HMCR550. Transient absorption measurements were performed with HMCR550 (50 µM) in 10 mM sodium phosphate buffer (excitation: 266 nm). See also Table S1 for τ_{cycl} calculation.



pH	measured τ_{cycl} (ms)	calculated τ_{cycl} (ms)
5	13	5.5
6	13	5.5
7	12	5.3
8	7.7	5.3
9	3.1	4.2
10	1.8	1.4
11	0.038	0.18
12	0.0050	0.018

Fig. S10 a) Chemical structure of 5-COOH-HMSiR600. b) Absorption/fluorescence spectra of 1 µM 5-COOH-HMSiR600 and pH dependency of absorbance at 595 nm and pH in 100 mM

sodium phosphate buffer containing 0.1% DMSO as cosolvent. c) Representative transient absorption decay measured at pH 7.0. d) Comparison of measured and calculated τ_{cycl} (lifetimes of the open form: the duration until the open form reverts to the closed form) of 5-COOH-HMSiR600. Transient absorption measurements were performed with 5-COOH-HMSiR600 (50 µM) in 10 mM sodium phosphate buffer (excitation: 266 nm). See also Table S1 for τ_{cycl} calculation.

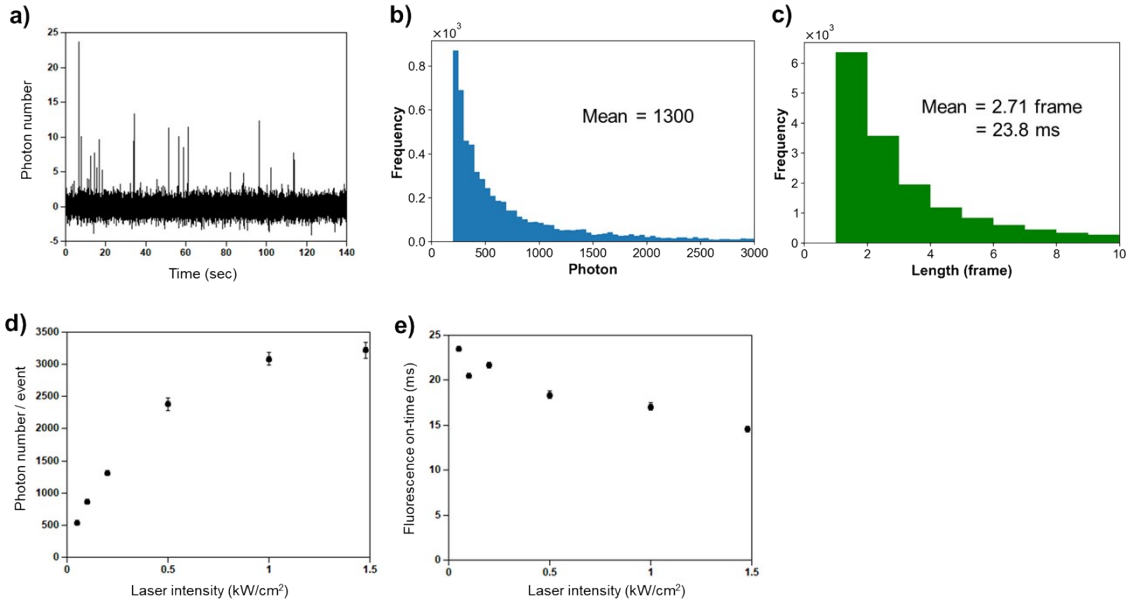


Fig. S11 Evaluation of blinking properties of HaloTag protein-conjugated 5-Halo-HMCR550 by single-molecule fluorescence imaging. a) Single-molecule fluorescence time trace. Excitation: 561 nm (100 W/cm^2). b) Distribution of photon number per switching event. c) Distribution of fluorescence-on time. b,c) Excitation: 561 nm (200 W/cm^2). 10000 frames were recorded at 8.8 ms/frame. d) Excitation intensity-dependency of photon number per switching event. e) Excitation intensity-dependency of fluorescence-on time. Mean ± s.e. (N = 2000-12000). Single-molecule imaging was performed in 10 mM sodium phosphate buffer (pH 7.4).

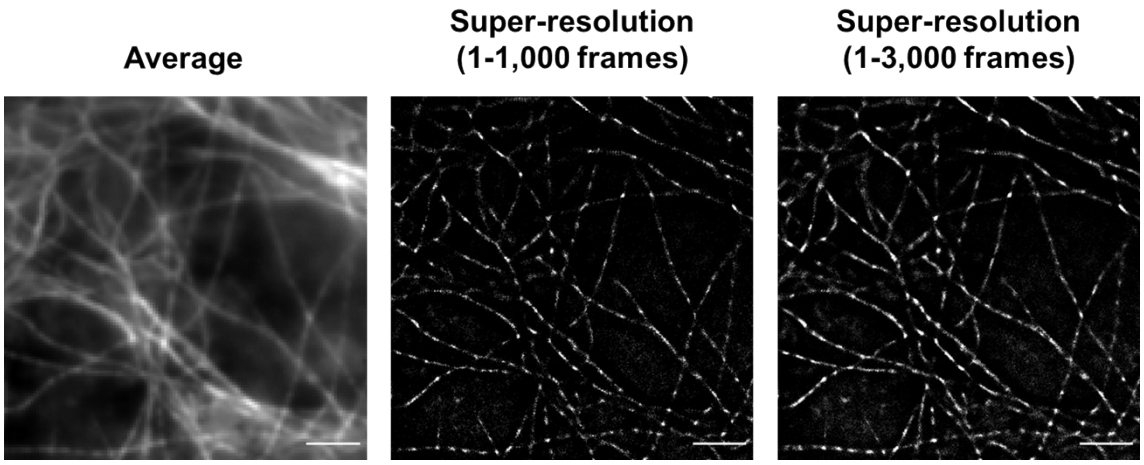


Fig. S12 Live-cell SMLM of Halo-tubulin fusion proteins stained with 5-Halo-HMCR550 in Vero cells. (left) Average and (middle, right) super-resolution images of microtubules. Super-resolution images were reconstructed from 1,000 or 3,000 frames (8.8 ms/frame). Excitation at 561 nm (300 W/cm^2). Scale bar, 3 μm.

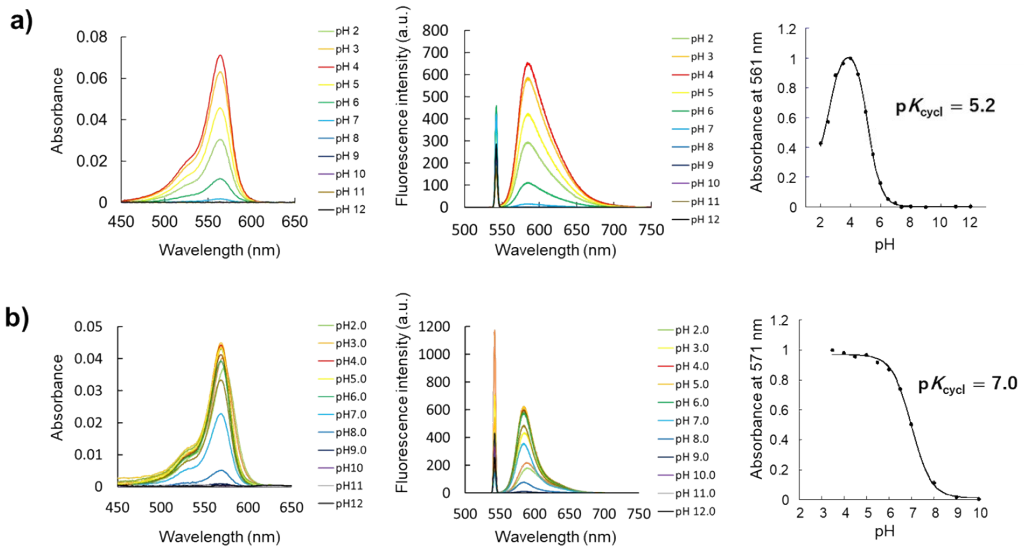


Fig. S13 (a) Absorption/fluorescence spectra of 5-Halo-HMCR550 at various pH values in 100 mM sodium phosphate buffer and correlation between pH and absorbance at 561 nm. (b) Absorption/fluorescence spectra of HaloTag-bound 5-Halo-HMCR550 at various pH values in 100 mM sodium phosphate buffer and correlation between pH and absorbance at 571 nm.

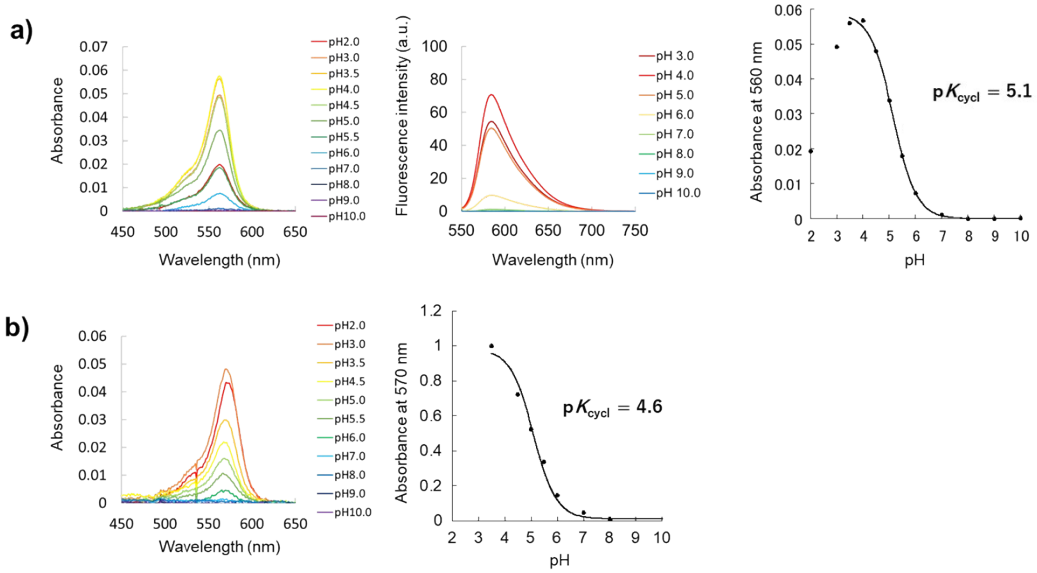


Fig. S14 a) Absorption/fluorescence spectra of 1 µM 4-Halo-HMCR550 in 100 mM sodium phosphate buffer containing 0.1% DMSO as cosolvent, and correlation between absorbance at 560 nm and pH. b) Absorption spectra of 1 µM HaloTag-bound 4-Halo-HMCR550 in sodium phosphate buffer containing 0.1% DMSO as cosolvent, correlation between absorbance at 570 nm and pH.

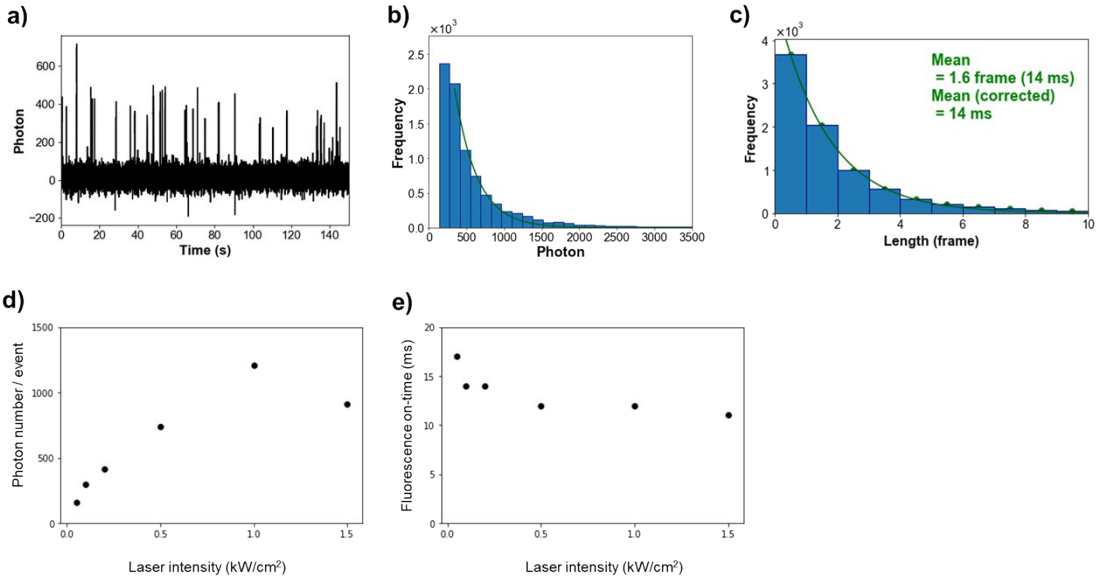


Fig. S15 Evaluation of blinking properties of HaloTag protein-conjugated 4-Halo-HMCR550 by single-molecule fluorescence imaging. a) Single-molecule fluorescence time trace. b) Distribution of photon number per switching event. c) Distribution of fluorescence-on time. a-c) Excitation: 561 nm (100 W/cm^2). 10000 frames were recorded at 8.8 ms/frame. d) Excitation intensity-dependency of photon number per switching event. e) Excitation intensity-dependency of fluorescence-on time. Mean ± s.e. (N = 2000-12000). Single-molecule imaging was performed in 10 mM sodium phosphate buffer (pH 7.4).

Table S1. Calculation values for estimating τ values and error from experimental values

Compound	pK_{aOH}	G_{open} (A) (hartree)	G_{TS} (A) (hartree)	G_{open} (B) (hartree)	G_{TS} (B) (hartree)	$\Delta G^{\ddagger}$ (A) (kJ/mol)	k_A (/sec)	$\Delta G^{\ddagger}$ (B) (kJ/mol)	k_B (/sec)	error(A) (kJ/mol)[a]	error(B) (kJ/mol)[a]
3MHMRG	15.4	-1300.522034	-1300.49616	-1299.476713	-1299.469941	67.9	7.5	17.8	4.7E+09	3	-2
M3MHMRG	15.4	-1339.812659	-1339.79049	-1338.740299	-1338.735269	58.2	383.0	13.2	3.0E+10	-3	-6
HMCR550	15.4	-1303.872039	-1303.846606	-1302.796479	-1302.786613	66.8	12.0	25.9	1.8E+08	1	3
HMTMCR	15.4	-1460.990851	-1460.957638	-1459.809477	-1459.799381	87.2	3.1E-03	26.5	1.4E+08		
HMCRB	15.4	-1618.154638	-1618.12218	-1616.879214	-1616.867575	85.2	7.0E-03	30.6	2.7E+07		
HMCR101	15.4	-1770.558027	-1770.519526	-1769.210994	-1769.192976	101.1	1.2E-05	47.3	3.1E+04		
HMSiR600	15.4	-1555.293161	-1555.270298	-1554.22557	-1554.215476	60.0	183.5	26.5	1.4E+08	-2	3
HMSiR	15.4	-1712.4111	-1712.384619	-1711.237785	-1711.228951	69.5	4.0	23.2	5.3E+08	-2	-3

[a] In order to calculate the average error between calculated and experimental values, the experimental τ values were fitted to an equation in Figure 1 to have $k_{A,exp}$, $k_{B,exp}$ values, which were the converted to $\Delta G^{\ddagger}(A)_{exp}$, $\Delta G^{\ddagger}(B)_{exp}$. The error between calculated and experimental values were calculated to be a few kJ/mol, which shows the reliability of our calculation method.

Table S2. Fluorescence quantum yield of HMR derivatives

	Φ_{fl}
HMRG	0.90[a]
3MHMRG	0.89[b,c]
M3MHMRG	0.88[c]
HMCR550	0.65[d]
HMSiR600	0.44[d,e]
5-COOH HMSiR600	0.44[d]
HMSiR	0.31[f,g]

[a]Data from ref.[1]. [b]Data from ref.[2]. [c]Measured in 0.1 M sodium phosphate buffer (pH 3.0). [d]Measured in 0.1 M sodium phosphate buffer (pH 4.0). [e]Data from ref.[3]. [f]Measured in 0.2 M sodium phosphate buffer (pH 4.5). [g]Data from ref.[4].

Table S3. Comparison of pK_{cycl} of 4(5)Halo-HMCR550 before and after binding to HaloTag protein.

	pK_{cycl}
4Halo-HMCR550	**5.1**
HaloTag-4Halo-HMCR550	**4.6**
5Halo-HMCR550	**5.2**
HaloTag-5Halo-HMCR550	**7.0**

Materials and methods

Reagents

General chemicals were of the best grade available, supplied by Aldrich Chemical Co., Ltd., Tokyo Chemical Industries, and Wako Pure Chemical, and were used without further purification. Special chemicals: dimethyl sulfoxide (from DOJINDO) used in spectroscopic analysis was of fluorometric grade. Other solvents were used after appropriate distillation or purification.

Instruments

NMR spectra were recorded on a JEOL JNM-LA300 instrument at 300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR or on a JEOL JNM-LA400 instrument at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. All chemical shifts (δ) are reported in ppm relative to internal standard tetramethylsilane (δ = 0.0 ppm), or relative to the signals of residual solvent $CDCl_3$ (7.26 ppm for ^{1}H, 77.16 ppm for ^{13}C), CD_3OD (3.31 ppm for ^{1}H, 49.00 ppm for ^{13}C) or DMSO-d_6 (2.50 ppm for ^{1}H, 39.52 ppm for ^{13}C), and coupling constants are given in Hz. Mass spectra were measured with a JEOL AccuTOF 4GLC-plus mass spectrometer or a Bruker micrOTOFII with electron spray ionization (ESI). HPLC analyses were performed on an Inertsil ODS-3 (4.5×250 mm) column (GL Sciences Inc.) using an HPLC system composed of a pump (PU-2080, JASCO) and a detector (MD-2015, JASCO). Preparative HPLC was performed with an Inertsil ODS-3 (10.0 mm ×250 mm) reverse-phase column (GL Science Inc.), using eluent A (H_2O containing 0.1% TFA (v/v)) and eluent B (CH_3CN with 20% H_2O), on a Jasco PU-1587S2 system. Silica gel column chromatography was performed with Wakogel C-200 (Wako, Japan), Chromatorex-NH (FujiSilysia Chemical Ltd., Kasugai, Japan), silica Gel 60 (Kanto Chemical Co. Inc., Tokyo, Japan), or silica gel 60N (Kanto Chemical Co. Inc., Tokyo, Japan).

LC analysis

Compound purity was confirmed by LC analysis with an ACUITY UPLC H-Class ultra-performance liquid chromatography mass spectroscopy (UPLC-MS) system (Waters) equipped with a column (Waters, ACQUITY UPLC BEH C18 1.7 mm), an autosampler (Waters, SMFTN; 186015017), a pump (Waters, QSM; 186015018), a PDA detector (Waters, eλ Detector; 186015033), and an MS detector (Waters, QDa; 186006511), using 0.1% formate solution (solution A) and acetonitrile (solution B) as eluents. A/B =95/5 to 5/95 (0 - 3.5 min), 5/95 (3.5 - 4.0 min), 5/95 to 95/5 (4.0 - 4.1 min), 95/5 (4.1 - 5.0 min). For M3MHMRG, m/z = 345.12; for HMCR550, m/z = 343.14; for 5-COOH-HMSiR600, m/z = 202.09; for 4-Halo-HMCR550, m/z = 592.20; for 5-Halo-HMCR550, m/z = 592.24

Computational details

We performed calculations using the Gaussian09[5] program. Stationary points were optimized without any symmetry assumptions, and tight convergence criteria were used. Bulk solvent effects in water were considered implicitly through the SMD polarizable continuum model[6]. A reactions were calculated at the B3LYP/6-31G(d,p) level, and B reactions were calculated at the CAM-B3LYP/6-31+G(d,p) level.

Measurements of photophysical properties

Compounds were dissolved in anhydrous DMSO to obtain 10 mM stock solutions. These stock solutions were diluted with 0.1 M sodium phosphate buffer to a final concentration of 1 µM. Absorption spectra were obtained with a UV-1800 UV/Vis spectrometer (Shimadzu) and fluorescence spectra were obtained with an F-4500 and F-7000 fluorescence spectrometer (Hitachi) at room temperature. Absolute fluorescence quantum yields were recorded on a Quantaurus QY C11347-1211 (Hamamatsu Photonics) at room temperature. To determine the pK_a values of compounds with n acid-base equilibria (n = 1 or 2), the pH profiles of absorbance (Abs) or fluorescence intensity (FI) were fitted to the following formula with KaleidaGraph software (version 4.1). The pK_a value was adopted as the pK_{cycl} value in the case of n = 1. I

$$Abs\ or\ FI = \frac{c_0 + \sum_{k=1}^{n} c_k \cdot 10^{k \cdot pH - \sum_{l=1}^{k} pK_{al}}}{1 + \sum_{k=1}^{n} 10^{k \cdot pH - \sum_{l=1}^{k} pK_{al}}}$$

(pK_{a1} < pK_{a2} < ••• < pK_{an}; c_n = constant).

Determination of τ_{cycl} by laser photolysis

Laser flash photolysis experiments were carried out by using a XeCl excimer laser (Lambda Physik, Lextra 50, pulse width 17 ns, 308 nm, 10 mJ/pulse) or the fourth harmonics (266 nm) of a Nd^{3+}: YAG laser (Spectra-Physics GCR-130, pulse width 6 ns, 10 mJ/pulse) as the excitation source. The transient signal was recorded on a digital storage oscilloscope (Tektronix, TDS-744 500 MHz 2G samples/s or TDS-540 500 MHz 1G sample/s). Experiments were performed at 22 °C. Compounds

were dissolved in 10 mM sodium phosphate buffer containing 1 % anhydrous DMSO, and absorbance at 308 nm was adjusted to be 0.2. The lifetime of the open form (τ_{cycl}: the duration until the open form reverts to the closed form) were determined by fitting the decay curves to a single exponential by using KaleidaGraph 4.5 or a self-written Python script implemented with the "curvefit" method in the "scipy.optimize" module.

Preparation of 4(5)-Halo-HMCR550 conjugates to HaloTag proteins.

Expression of His-tagged HaloTag protein in BL21(DE3) transformed with Halo-Histag-pET3a plasmid was induced by isopropyl ß-D-1-thiogalactopyranoside (IPTG) followed by culture until the optical density at 600 nm reached 0.6 or with the Overnight Express system. The protein was extracted and purified with QIAexpress Ni-NTA Fast Start (QIAGEN) according to the manufacturer's protocol. The purified HaloTag protein was labeled with 4(5)-Halo-HMCR550 in 50 mM Tris-HCl buffer solution (pH 7.5) containing 100 mM NaCl, 0.1% Tween 20, and 1 mM DTT at 37 °C for 60 min. The labeled protein was purified through a PD-10, MiniTrapTM G-25, or PD MiniTrapTM G-25 column (GE Healthcare) and then through an Amicon Ultra-4 10K centrifugal unit (Merck). The obtained protein solution was analyzed by SDS-PAGE to evaluate the degree of labeling, and used for single-molecule fluorescence measurements under a microscope.

Microscope

SMLM imaging was carried out using an N-STORM system (Nikon) equipped with the following devices:

- Inverted fluorescence microscope (Eclipse Ti-E; Nikon)
- Oil-immersion objective (CFI Apo TIRF 100X Oil, NA 1.49; Nikon)
- Irradiation laser: 561 nm laser (Sapphire 561 LP; Coherent)
- Cooled electron-multiplying charge-coupled device (EMCCD) camera (iXon3 DU897; Andor).
- Software for system control and image analysis (NIS-Elements Advanced Research; Nikon)

During data acquisition, a Perfect Focus System was used to maintain a constant focal plane.

Sample preparation for single-molecule fluorescence imaging.

The single-molecule blinking properties of 4(5)-Halo-HMCR550 conjugated to HaloTag proteins were evaluated by using a flow cell consisting of two glass coverslips separated by double-sided tape. Coverslips were cleaned beforehand by sonication in Milli-Q water for 10 min, in 99% EtOH for 10 min, and then in 10 N KOH aq. for 10 min. Then, the sonicated coverslips were soaked in 10 N KOH aq. overnight, washed with Milli-Q water, and blow-dried with compressed air from a gas duster (HFC-152a) before assembly of the flow cell. A diluted sample solution was loaded into a flow cell, so that the fluorophore-protein conjugates were adsorbed on the surface of the flow cell at a low density. The flow cell was rinsed with 10 mM sodium phosphate buffer (pH 7.4) to remove excess fluorophore. HaloTag conjugates of 4(5)-Halo-HMCR550 were imaged in 10 mM sodium phosphate buffer at pH 7.4.

Culturing and preparing cells

Green monkey kidney Vero cells (JCRB0111) were obtained from the Japanese Collection of Research Bioresources (JCRB) cell bank. Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose) containing L-glutamine (Wako Pure Chemical), supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (PS) solution (Gibco) at 37 °C in humidified air containing 5% CO_2. To minimize the background fluorescence, phenol red-free medium (Gibco) was preferred. Cells were regularly passaged using TrypLE Express not containing phenol red (Gibco).

For imaging experiments, cells were seeded into 8-well Lab Tek II Chambered Coverglass (No. 1.5 borosilicate; Nunc). To avoid non-specific adsorption of fluorophores, the coating process was preferably done by using a solution of 2 M glycine (Wako Pure Chemical) or 0.01% poly-L-lysine (Sigma) for 30-60 min, followed by washing with phosphate-buffered saline (PBS; Gibco) several times.

Labelling procedure of β-tubulin-Halo in live Vero cells

1. The day before imaging, transfect Vero cells transiently with β-tubulin-Halo plasmid in the presence of a transfection reagent (X-treamGENE HP DNA Transfection Reagent) according to the manufacturer's protocol. Incubate the cells at 37 °C under 5% CO_2 overnight.
2. Prepare stock solutions of 4(5)-Halo-HMCR550 in dimethyl sulfoxide (DMSO) at 1 mM.

3. After washing the cells once with culture medium, incubate the cells in culture medium containing 1 μM 4(5)-Halo-HMCR550 for 40-60 min, followed by washing 3-4 times.

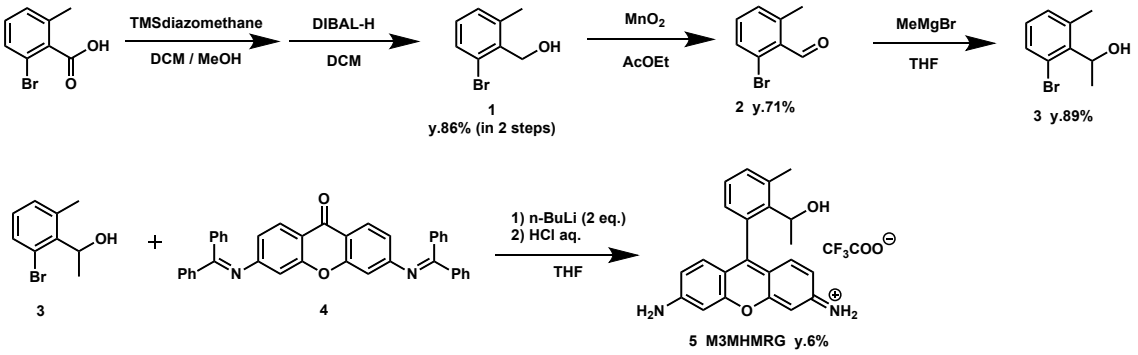
Live-cell SMLM imaging in Vero cells

1. Renew the medium of the sample.
2. Carry out data acquisition for SMLM with the N-STORM system at room temperature (23 °C). 4(5)-Halo-HMCR550 is excited with the 561 nm laser in a total-internal-reflection or oblique incidence geometry.

• For fast time-lapse SMLM, record typically tens of thousands of consecutive frames of images at 8.8-15 ms/frame, applying continuous illumination at 561 nm with a relatively low (200-400 W/cm^2) intensity.

3. Analyze the raw images to reconstruct super-resolution images with NIS-Elements Advanced Research software implemented with an N-STORM module.

Synthesis



Scheme S1. Synthesis of M3MHMRG.

Compound 1

2-Bromo-6-methylbenzoic acid (2050 mg, 9.5 mmol) was dissolved in CH_2Cl_2 (20 mL) and methanol (6 mL). Trimethylsilyldiazomethane 10% in hexane solution (18.6 mL, 11.2 mmol) was added and the solution was stirred at r.t. for 10 minutes. The reaction was quenched with AcOH and the mixture was extracted with CH_2Cl_2 from saturated $NaHCO_3$ aq.. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was dissolved in anhydrous CH_2Cl_2 (20 mL), and the resulting solution was stirred at 0˚C under an Ar atmosphere. Diisobutylaluminium hydride (18.6 mL, 18.6 mmol) was added and stirring was continued at r.t. under an Ar atmosphere for 2 hours. The reaction was quenched with methanol and saturated potassium sodium tartrate aq. then the mixture was extracted with CH_2Cl_2. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt = 9/1 to 7/3) to give compound 1 as a colorless solid (1640 mg, 86%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.46 (s, 3H), 4.82 (s, 2H), 7.04 (dd, 1H, *J* = 8.1 Hz, 8.1 Hz), 7.12 (d, 1H, *J* = 8.1 Hz), 7.40 (d, 1H, *J* = 8.1 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 20.0, 62.1, 125.4, 129.4, 129.9, 130.6, 137.4, 139.7. MS peak was not detected by our ESI-MS system.

Compound 2

Compound 1 (1600 mg, 8.0 mmol) was dissolved in anhydrous AcOEt (40 mL). Manganese dioxide (6919 mg, 80.0 mmol) was added and the mixture was stirred at 70˚C for 18 hours. The mixture was filtered with celite and evaporated. The residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt = 10/0 to 9/1) to give compound 2 as a pale yellow solid (1120 mg, 71%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.55 (s, 3H), 7.18 (d, 1H, *J* = 7.2 Hz), 7.22 (dd, 1H,

J = 7.2 Hz, 7.6 Hz), 7.48 (d, 1H, *J* = 7.6 Hz), 10.5 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 31.7, 128.4, 131.5, 131.7, 131.8, 133.7, 142.7, 194.6. MS peak was not detected by our ESI-MS system.

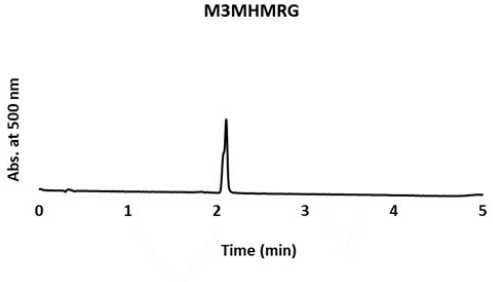
Compound 3

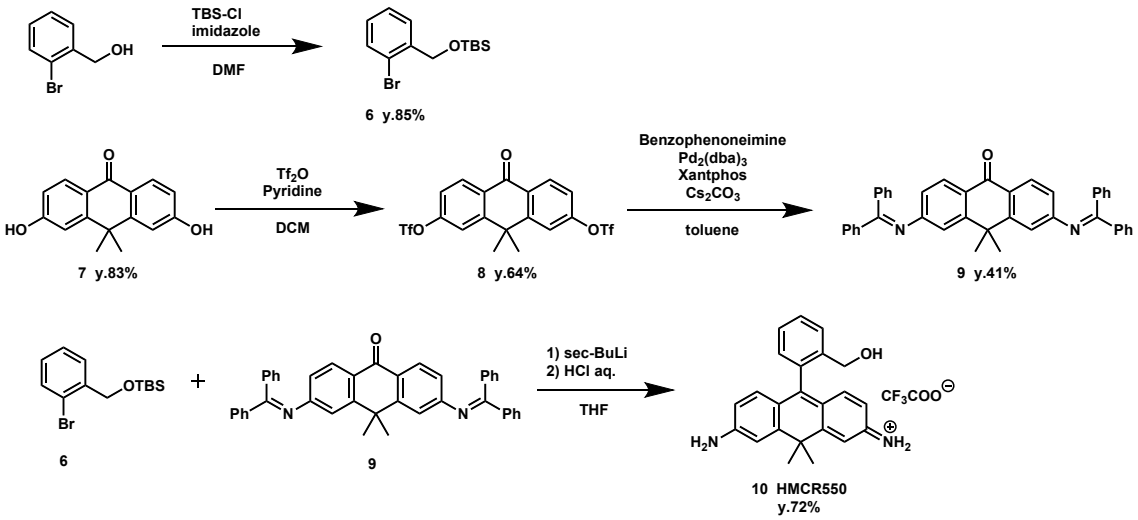
To a flame-dried flask flushed with Ar, compound 2 (400 mg, 2.0 mmol) and anhydrous THF (10 mL) were added. The mixture was cooled to -78˚C and then 3 M methylmagnesium bromide (0.7 mL, 2.0 mmol) was added to it. The mixture was stirred at r.t. for 1 hour, then the reaction was quenched with H_2O, and the mixture was extracted with CH_2Cl_2 from saturated $NaHCO_3$ aq.. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt = 9/1 to 7/3) to give compound 3 as a colorless solid (384 mg, 89%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.56 (d, 3H, *J* = 6.8 Hz), 2.49 (s, 3H), 2.49 (s, 1H), 5.45-5.52 (m, 1H), 7.00 (dd, 1H, *J* = 7.6 Hz, 7.6 Hz), 7.08 (d, 1H, *J* = 7.6 Hz), 7.37 (d, 1H, *J* = 7.6 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.8, 21.5, 70.2, 122.7, 128.3, 131.3, 131.4, 138.7, 140.7. MS peak was not detected by our ESI-MS system.

Compound 4 was prepared according to the literature[7].

Compound 5 (M3MHMRG)

To a flame-dried flask flushed with Ar, compound 3 (387 mg, 1.8 mmol) and anhydrous THF (13 mL) were added. The mixture was cooled to -78˚C and then 1 M *n*-BuLi (2.0 mL, 3.2 mmol) was added to it. Compound 4 (100 mg, 0.2 mmol) in anhydrous THF (4 mL) was further added. The mixture was stirred at r.t. for 1 hour, then the reaction was quenched with 1 N HCl aq., and the mixture was extracted with CH_2Cl_2 from saturated $NaHCO_3$ aq.. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by preparative HPLC under the following conditions: A/B = 80/20 (0 min) to 0/100 (45 min) linear gradient (solvent A: H_2O, 0.1% TFA; solvent B: acetonitrile/H_2O = 80/20, 0.1% TFA). Compound 5 was obtained as a dark violet solid (4 mg, 6%). ^{1}H NMR (400 MHz, CD_3OD+NaOD): δ 1.57 (d, 3H, *J* = 6.4 Hz), 2.41 (s, 3H), 5.48 (q, 1H, *J* = 6.4 Hz), 6.32 (d, 1H, *J* = 7.6 Hz), 6.38-6.46 (m, 4H), 6.57 (d, 1H, *J* = 6.0 Hz), 6.71 (d, 1H, *J* = 8.4 Hz), 7.12-7.17 (m, 2H). HRMS (ESI$^+$): Calcd for $[M+H]^+$, 345.16030, Found, 345.15620 (-4.10 mmu).





Scheme S2. Synthesis of HMCR550.

Compound 6

2-Bromobenzyl alcohol (2000 mg, 10.7 mmol) was dissolved in anhydrous DMF (40 mL). tert-Butyldimethylchlorosilane (2412 mg, 16.0 mmol) and imidazole (2185 mg, 32.1 mmol) were added and the solution was stirred at r.t. under an Ar atmosphere for 4 hours. The mixture was extracted with *n*-hexane. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by flash column chromatography (silica gel, *n*-hexane) to give compound 6 as a colorless liquid (2750 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.14 (s, 6H), 0.97 (s, 9H), 4.74 (s, 2H), 7.11 (dd, 1H, *J* = 8.0 Hz, 8.0 Hz), 7.33 (dd, 1H, *J* = 8.0 Hz, 8.0 Hz), 7.49 (d, 1H, *J* = 8.0 Hz), 7.56 (d, 1H, *J* = 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ -5.2, 18.5, 26.0, 64.7, 121.1, 127.4, 127.6, 128.2, 132.1, 140.4. MS peak was not detected by our ESI-MS system.

Compound 7 as prepared according to the literature[8].

Compound 8

Compound 7 (1750 mg, 6.9 mmol) and pyridine (2.2 mL, 27.5 mmol) were dissolved in anhydrous CH_2Cl_2 (40 mL) and the mixture was stirred at 0˚C. Then, trifluoromethanesulfonic anhydride (4.5 mL, 27.5 mmol) was added, and stirring was continued for 16 hours. The reaction was quenched with H_2O and the mixture was extracted with CH_2Cl_2. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt = 9/1 to 7/3) to give compound 8 as a colorless solid (2290 mg, 64%). ^{1}H NMR (400

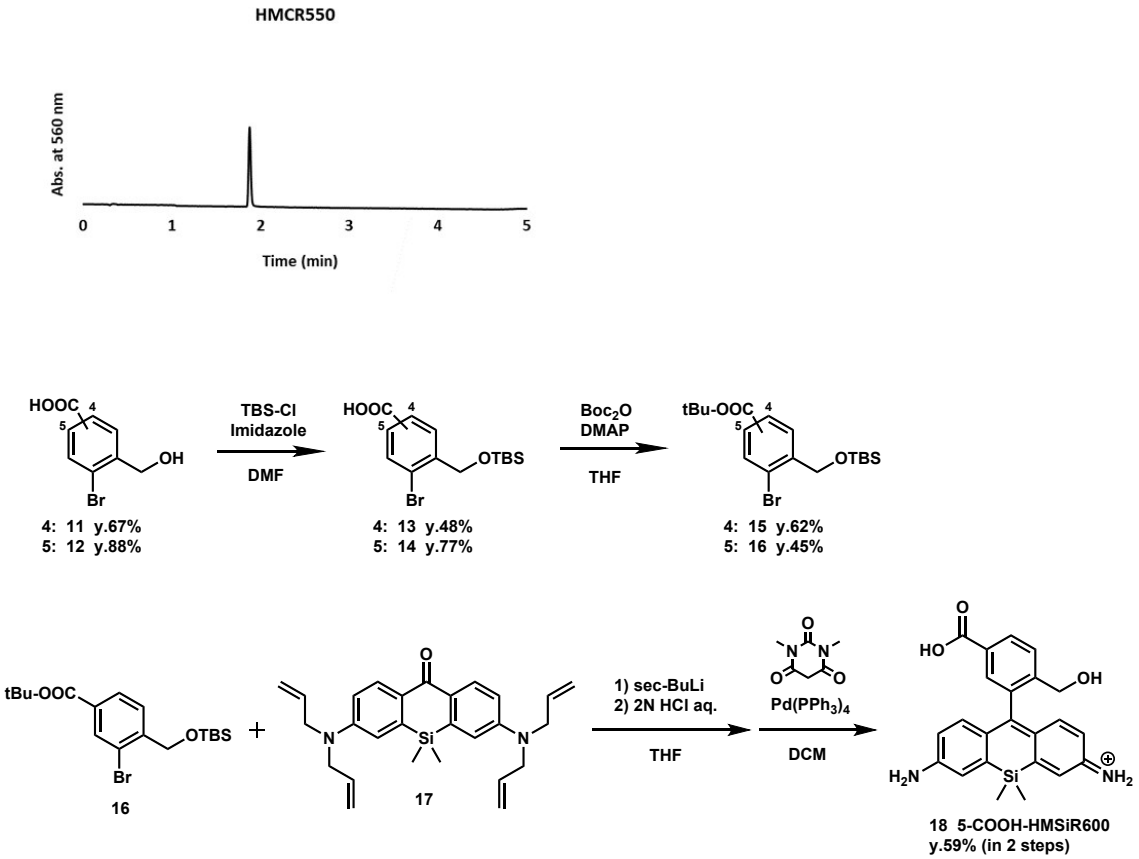
MHz, $CDCl_3$): δ 1.77 (s, 6H), 7.37 (dd, 2H, *J* = 2.4 Hz, 8.8 Hz), 7.57 (d, 2H, *J* = 2.4 Hz), 8.45 (d, 2H, *J* = 8.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 32.9, 38.9, 118.8(q, *J* = 320.8 Hz), 119.9, 120.5, 129.3, 130.8, 152.6, 153.3, 180.8.

Compound 9

Compound 8 (2270 mg, 4.4 mmol), benzophenone imine (7938 mg, 43.8 mmol), $Pd_2(dba)_3$ (403 mg, 0.44 mmol), xantphos (633 mg, 1.1 mmol) and Cs_2CO_3 (14271 mg, 43.8 mmol) were dissolved in deaerated dioxane (20 mL) and the solution was stirred at 80˚C under an Ar atmosphere for 15 hours. The mixture was extracted with CH_2Cl_2 and the organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt = 8/2 to 6/4) and washed with hexane/AcOEt = 2/1 to give compound 9 as a yellow solid (1050 mg, 41%). ^1H NMR (400 MHz, $CDCl_3$): δ 1.25 (s, 6H), 6.78 (d, 2H, *J* = 2.0 Hz), 6.82 (dd, 2H, *J* = 8.0 Hz, 2.0 Hz), 7.09-7.11 (m, 4H), 7.21-7.23 (m, 6H), 7.38-7.52 (m, 6H), 7.79 (d, 2H, *J* = 7.6 Hz), 8.14 (d, 2H, *J* = 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 32.5, 37.7, 118.6, 120.3, 125.7, 128.2, 128.4, 128.4, 128.9, 129.4, 129.6, 131.3, 135.9, 139.1, 151.3, 155.9, 169.2, 182.4. HRMS (ESI^+): Calcd for $[M+H]^+$, 581.25929, Found, 581.25803 (-1.25 mmu)

Compound 10 (HMCR550)

To a flame-dried flask flushed with Ar, compound 6 (512 mg, 1.7 mmol) and anhydrous THF (15 mL) were added. The mixture was cooled to -80˚C and then 1 M *sec*-BuLi (1.3 mL, 1.3 mmol) was added to it. Compound 9 (100 mg, 0.17 mmol) in anhydrous THF (5 mL) was further added, and the mixture was stirred at r.t. for 1 hour. The reaction was quenched with 2 N HCl aq. and the mixture was extracted with CH_2Cl_2 from saturated $NaHCO_3$ aq.. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by preparative HPLC under the following conditions: A/B = 80/20 (0 min) to 0/100 (30 min) linear gradient (solvent A: H_2O, 0.1% TFA; solvent B: acetonitrile/H_2O = 80/20, 0.1% TFA). Compound 10 was obtained as an orange solid (42 mg, 72%). ^1H NMR (400 MHz, CD_3OD+NaOD): δ 1.65 (s, 3H), 1.72 (s, 3H), 5.28 (s, 2H), 6.49 (dd, 2H, *J* = 8.4 Hz, 2.0 Hz), 6.61 (d, 1H, *J* = 8.0 Hz), 6.62 (d, 2H, *J* = 8.4 Hz), 6.94 (d, 2H, *J* = 2.0 Hz), 7.16 (dd, 2H, *J* = 8.0 Hz, 8.0 Hz), 7.28 (dd, 2H, *J* = 8.0 Hz, 8.0 Hz), 7.36 (d, 1H, *J* = 8.0 Hz). ^{13}C NMR (100 MHz, CD_3OD+NaOD): δ 32.9, 33.8, 37.3, 71.8, 88.5, 111.9, 114.5, 120.5, 123.3, 127.2, 127.3, 127.8, 129.7, 138.3, 145.1, 147.0, 147.8. HRMS (ESI^+): Calcd for $[M+H]^+$, 343.18049, Found, 343.18013 (-0.36 mmu).



Scheme S3. Synthesis of 5-COOH-HMSiR600.

Compound 11 was prepared according to the literature[4].

Compound 12 was prepared in the same way as compound 11, according to the literature[4]. ^{1}H NMR (400 MHz, acetone-d6) δ:8.12 (d, 1H, J = 1.4 Hz), 8.02 (dd, 1H, J = 8.0, 1.6 Hz), 7.75 (d, 1H, J = 7.8 Hz), 4.71 (s, 2H) ^{13}C NMR (400 MHz, acetone-d6) δ: 165.5, 146.4, 133.1, 130.8, 128.7, 127.8, 120.8, 63.3. MS peak was not detected by our ESI-MS system.

Compound 13

Compound 11 (3600 mg, 15.6 mmol) was dissolved in anhydrous DMF (30 mL). tert-Butyldimethylchlorosilane (4696 mg, 31.2 mmol) and imidazole (3182 mg, 46.8 mmol) were added and the solution was stirred at r.t. under an Ar atmosphere for 6 hours. The mixture was evaporated. The residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt = 8/2 to 6/4) to give compound 13 as a colorless solid (2169 mg, 86%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.07 (s, 6H), 0.89 (s, 9H), 4.69 (s, 2H), 7.68 (dd, 1H, *J* = 8.4 Hz, 2.0 Hz), 7.72 (d, 1H, *J* = 2.0 Hz), 8.06

(d, 1H, *J* = 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ -4.9, 18.5, 26.3, 64.4, 126.2, 129.0, 130.0, 130.8, 133.1, 140.7, 167.2. MS peak was not detected by our ESI-MS system.

Compound 14

Compound 12 (6000 mg, 26.0 mmol) was dissolved in anhydrous DMF (40 mL). tert-Butyldimethylchlorosilane (5900 mg, 39.0 mmol) and imidazole (3500 mg, 51.9 mmol) were added and the solution was stirred at r.t. under an Ar atmosphere for 7 hours. The mixture was evaporated. The residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt(2% AcOH) = 100/0 to 85/15) to give compound 14 as a colorless solid (6915 mg, 77%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 8.23 (d, 1H, *J* = 1.8 Hz), 8.07 (dd, 1H, *J* = 8.0, 1.6 Hz), 7.68 (d, 1H, *J* = 8.2 Hz), 4.77 (s, 2H), 0.97 (s, 9H), 0.15 (s, 6H). ^{13}C-NMR (400 MHz, $CDCl_3$) δ: 170.8, 146.8, 133.8, 129.3, 129.2, 127.5, 120.8, 64.7, 26.0, 18.5, -5.3. MS peak was not detected by our ESI-MS system.

Compound 15

Compound 13 (2800 mg, 8.1 mmol) was dissolved in anhydrous THF (30 mL). Di-tert-butyl pyrocarbonate (4425 mg, 20.3 mmol) and *N,N*-dimethyl-4-aminopyridine (495 mg, 4.1 mmol) were added and the solution was refluxed at 80˚C for 20 hours. Then the reaction was quenched with brine, and the mixture was extracted with CH_2Cl_2 and AcOEt. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt = 10/0 to 9/1) to give compound 15 as a colorless liquid (2000 mg, 62%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.13 (s, 6H), 0.97 (s, 9H), 1.57 (s, 9H), 4.72 (s, 2H), 7.52 (d, 1H, *J* = 8.4 Hz), 7.72 (dd, 1H, *J* = 8.4 Hz, 2.0 Hz), 8.16 (d, 1H, *J* = 2.0 Hz). MS peak was not detected by our ESI-MS system.

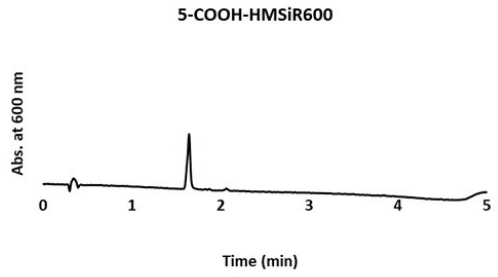
Compound 16

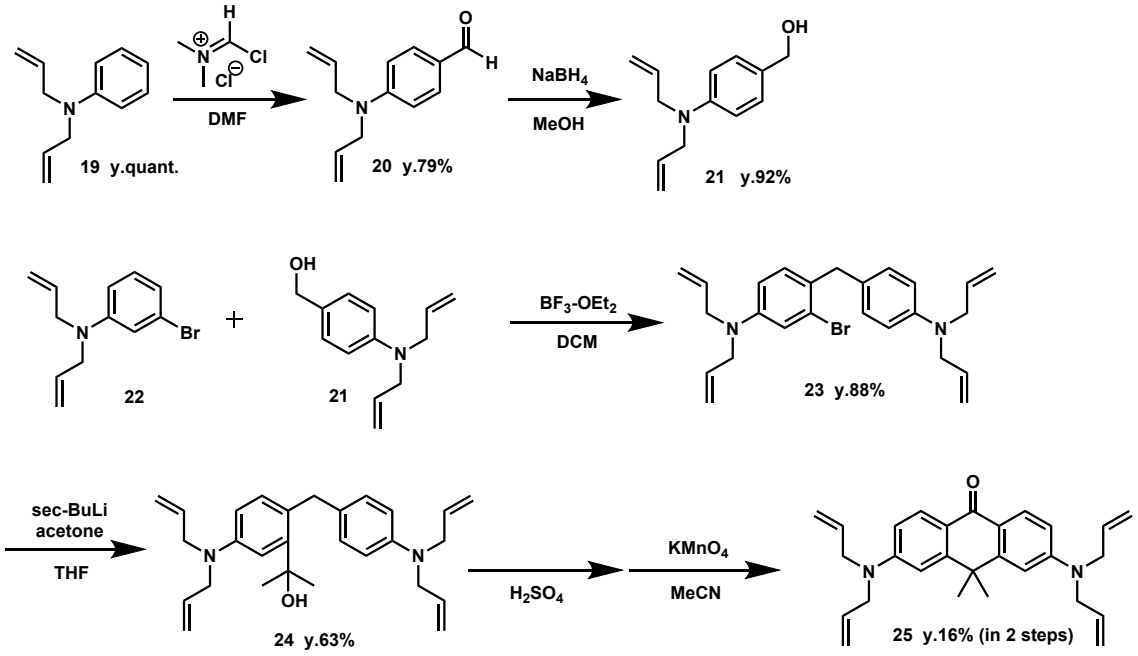
Compound 14 (2417 mg, 7.0 mmol) was dissolved in anhydrous THF (20 mL). Di-tert-butyl pyrocarbonate (3055 mg, 14.0 mmol) and *N,N*-dimethyl-4-aminopyridine (171 mg, 1.4 mmol) were added and the solution was refluxed at 100˚C for 22 hours. Then the reaction mixture was evaporated. The residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt = 10/0 to 9/1) to give compound 16 as a colorless liquid (1227 mg, 45%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 8.08 (d, 1H, *J* = 1.4 Hz), 7.94 (dd, 1H, *J* = 8.2, 1.4 Hz), 7.60 (d, 1H, *J* = 8.2 Hz), 4.74 (s, 2H), 1.58 (s, 9H), 0.96 (s, 9H), 0.13 (s, 6H). ^{13}C-NMR (400 MHz, $CDCl_3$) δ: 164.5, 145.0, 133.0, 132.2, 128.4, 127.2, 120.6, 81.5, 64.7, 28.2, 26.0, 18.5, -5.3. MS peak was not detected by our ESI-MS system.

Compound 17 was prepared according to the literature[9].

Compound 18 (5-COOH-HMSiR600)

To a flame-dried flask flushed with Ar, compound 16 (1160 mg, 3.0 mmol) and anhydrous THF (20 mL) were added. The mixture was cooled to -78˚C and then 1 M *sec*-BuLi (3.0 mL, 3.0 mmol) was added to it. Compound 17 (183 mg, 0.43 mmol) in anhydrous THF (5 mL) was further added. The mixture was stirred at r.t. for 2 hours, then the reaction was quenched with 2 N HCl aq., and the mixture was extracted with AcOEt. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was dissolved in dehydrated CH_2Cl_2 (20 mL), and 1,3-dimethylbarbituric acid (320 mg, 2.1 mmol) and $Pd(PPh_3)_4$ (100 mg, 0.09 mmol) were added. The resulting solution was stirred at 40˚C under an Ar atmosphere for 21 hours. The mixture was evaporated. The residue was purified by preparative HPLC under the following conditions: A/B = 90/10 (0 min) to 0/100 (30 min) linear gradient (solvent A: H_2O, 0.1% TFA; solvent B: acetonitrile/H_2O = 80/20, 0.1% TFA). Compound 18 was obtained as a blue solid (30.5 mg, 59%). ^{1}H NMR (400 MHz, CD_3OD): δ 7.94 (dd, J = 7.9, 1.4 Hz, 1H), 7.67 (d, J = 2.5 Hz, 2H), 7.61 (d, J = 8.7 Hz, 2H), 7.55 (d, J = 1.4 Hz, 1H), 7.50 (d, J = 8.0 Hz, 1H), 7.33 (dd, J = 8.6, 2.5 Hz, 2H), 5.69 (s, 2H), 0.81 (s, 3H), 0.60 (s, 3H). HRMS (ESI+): Calcd for[M^+], 403.14779, Found, 403.14745(-0.35mmu).





Scheme S4. Synthesis of compound 25.

Compound 19 was prepared according to the literature[10].

Compound 20

Vilsmeier reagent (7.4 g, 57.7 mmol) was dissolved in anhydrous DMF (40 mL) and the solution was stirred at 0˚C under an Ar atmosphere. Then, compound 19 (10.0 g, 10.9 mL, 57.7 mmol) was added, and stirring was continued at r.t. for 20 hours. The reaction was quenched with saturated $NaHCO_3$ aq. and the mixture was extracted with CH_2Cl_2. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt = 9/1 to 2/1) to give compound 20 as a colorless liquid (9.14 g, 79%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.99 (d, 4H, *J* = 5.6 Hz), 5.12-5.20 (m, 4H), 5.79-5.87 (m, 2H), 6.69 (d, 2H, *J* = 9.2 Hz), 7.69 (d, 2H, *J* = 9.2 Hz), 9.71 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 52.8, 111.5, 116.8, 125.7, 132.1, 132.3, 153.3, 190.3. MS peak was not detected by our ESI-MS system.

Compound 21

Compound 20 (8000 mg, 39.7 mmol) was dissolved in anhydrous methanol (50 mL) and stirred at 0˚C. Sodium tetrahydroborate (1654 mg, 43.7 mmol) was added and stirring was continued at r.t. for 4 hours. The reaction was quenched with H_2O and the mixture was extracted with CH_2Cl_2. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by flash

column chromatography (silica gel, *n*-hexane/AcOEt = 2/1 to 1/1) to give compound 21 as a colorless liquid (7450 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.92 (d, 4H, *J* = 4.0 Hz), 4.53 (s, 2H), 5.14-5.19 (m, 4H), 5.80-5.89 (m, 2H), 6.67 (d, 2H, *J* = 9.2 Hz), 7.19 (d, 2H, *J* = 9.2 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 52.9, 65.4, 112.4, 116.1, 128.7, 128.8, 133.9, 148.5. HRMS (ESI^+): Calcd for $[M+H]^+$, 204.13884, Found, 204.13520 (-3.64 mmu).

Compound 22 was prepared according to the literature[9].

Compound 23

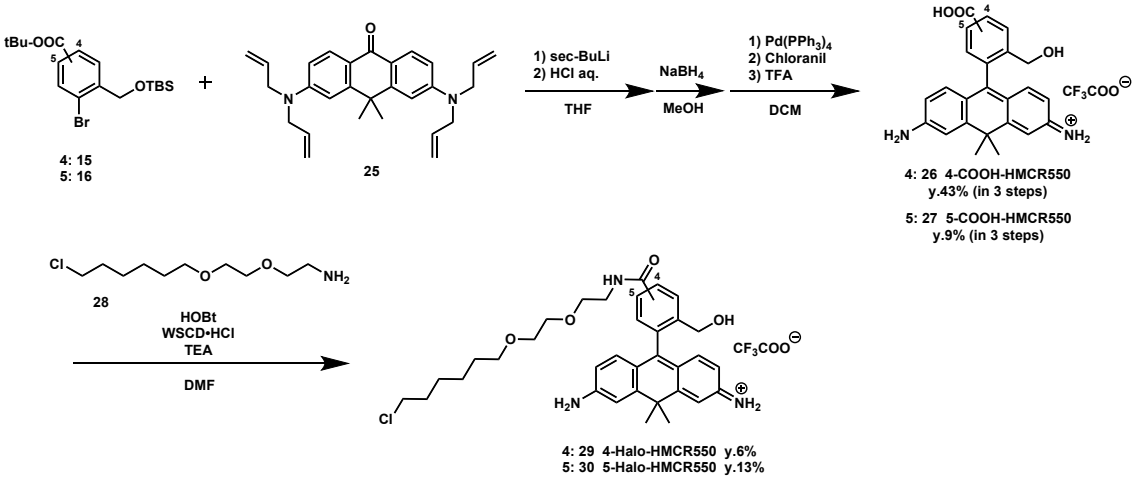
Compound 22 (2522 mg, 10.0 mmol) and compound 21 (2030 mg, 10.0 mmol) were dissolved in anhydrous CH_2Cl_2 (20 mL) and the solution was stirred at 0˚C. Boron trifluoride ethyl ether complex (2.5 mL, 20.0 mmol) was added, and stirring was continued at r.t. for 22 hours. The reaction was quenched with saturated $NaHCO_3$ aq. and the mixture was extracted with CH_2Cl_2. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt = 10/0 to 8/2) to give compound 23 as a colorless liquid (3870 mg, 88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.85-3.90 (m, 10H), 5.12-5.19 (m, 8H), 5.77-5.90 (m, 4H), 6.55 (dd, 1H, *J* = 8.4 Hz, 2.8 Hz), 6.63 (d, 2H, *J* = 8.4 Hz), 6.87 (d, 1H, *J* = 2.8 Hz), 6.93 (d, 1H, *J* = 8.4 Hz), 7.02 (d, 2H, *J* = 8.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 39.7, 52.8, 52.9, 111.8, 112.5, 116.0, 116.1, 116.3, 125.5, 128.4, 128.6, 129.6, 131.1, 133.6, 134.4, 147.1, 148.1. HRMS (ESI^+): Calcd for $[M+H]^+$, 437.15924, 439.15719, Found, 437.16055, 439.15909 (+1.32 mmu, +1.90 mmu)

Compound 24

To a flame-dried flask flushed with Ar, compound 23 (1800 mg, 4.1 mmol) and anhydrous THF (15 mL) were added. The mixture was cooled to -78˚C and then 1 M *sec*-BuLi (4.1 mL, 4.1 mmol) was added to it. Acetone (0.6 mL, 8.2 mmol) was further added, and the mixture was stirred at r.t. for 3 hours. The reaction was quenched with H_2O and the mixture was extracted with CH_2Cl_2 from saturated $NaHCO_3$ aq.. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt = 10/0 to 8/2) to give compound 24 as a colorless solid (1073 mg, 63%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.60 (s, 6H), 1.76 (s, 1H), 3.87-3.91 (m, 8H), 4.16 (s, 2H), 5.11-5.22 (m, 8H), 5.79-5.92 (m, 4H), 6.56 (d, 1H, *J* = 8.0 Hz), 6.61 (d, 2H, *J* = 7.2 Hz), 6.82 (s, 1H), 6.93-6.97 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 31.8, 38.0, 53.0, 53.2, 74.3, 110.2, 111.3, 112.6, 116.0, 116.2, 126.5, 129.4, 130.9, 134.0, 134.4, 134.6, 146.6, 146.9, 146.9. HRMS (ESI^+): Calcd for $[M+H]^+$, 417.29004, Found, 417.28930 (-0.74 mmu).

Compound 25

Compound 24 (8900 mg, 21.4 mmol) was dissolved in 95% H_2SO_4 (10 mL) and the solution was stirred at 0˚C for 10 minutes. The reaction was quenched with saturated Na_2CO_3 aq. and the mixture was extracted with CH_2Cl_2. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was dissolved in acetonitrile (120 mL) and the solution was stirred at 0˚C. $KMnO_4$ (10128 mg, 64.1 mmol) was added portionwise, then the mixture was stirred at r.t. for 2 hours, and the reaction was quenched with methanol. The mixture was filtered through Celite, and the filtrate was evaporated. The residue was purified by flash column chromatography (silica gel, CH_2Cl_2/methanol = 100/0 to 97/3) to give compound 25 as a light yellow solid (1420 mg, 16%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.63 (s, 6H), 4.02 (d, 8H, *J* = 2.8 Hz), 5.20-5.23 (m, 8H), 5.84-5.93 (m, 4H), 6.72 (dd, 2H, *J* = 2.0 Hz, 8.8 Hz), 6.76 (d, 2H, *J* = 2.0 Hz), 8.20 (d, 2H, *J* = 8.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 33.6, 38.1, 53.0, 108.5, 111.1, 116.6, 120.3, 129.2, 133.3, 151.8, 152.3, 181.1. HRMS (ESI$^+$): Calcd for $[M+H]^+$, 413.25929, Found, 413.25696(-2.23mmu)



Scheme S5. Synthesis of 4(5)-Halo-HMCR550.

Compound 26 (4-COOH-HMCR550)

To a flame-dried flask flushed with Ar, compound 15 (482 mg, 1.2 mmol) and anhydrous THF (10 mL) were added. The mixture was cooled to -78˚C and then 1 M *sec*-BuLi (1.2 mL, 1.2 mmol) was added to it. Compound 25 (100 mg, 0.24 mmol) in anhydrous THF (4 mL) was further added. The mixture was stirred at r.t. for 1 hour, then the reaction was quenched with 2 N HCl aq., and the mixture was extracted with CH_2Cl_2 from saturated $NaHCO_3$ aq.. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was dissolved in methanol (5 mL), and the solution was stirred at 0˚C. Sodium tetrahydroborate (27 mg, 0.72 mmol) was added and stirring was

continued at r.t. for 10 minutes. The reaction was quenched with H_2O and the mixture was extracted with AcOEt. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was dissolved in dehydrated CH_2Cl_2 (20 mL), and 1,3-dimethylbarbituric acid (187 mg, 1.2 mmol) and $Pd(PPh_3)_4$ (55 mg, 0.05 mmol) were added. The resulting solution was stirred at 40˚C under an Ar atmosphere for 20 hours. The mixture was evaporated and dissolved in CH_2Cl_2 (5 mL). Then chloranil (118 mg, 0.48 mmol) and TFA (5 mL) were added, and stirring was continued at r.t. for 10 minutes. The mixture was evaporated. The residue was purified by preparative HPLC under the following conditions: A/B = 80/20 (0 min) to 0/100 (30 min) linear gradient (solvent A: H_2O, 0.1% TFA; solvent B: acetonitrile/H_2O = 80/20, 0.1% TFA). Compound 26 was obtained as a violet solid (40 mg, 43%). ^{1}H NMR (400 MHz, CD_3OD+NaOD): δ 1.64 (s, 3H), 1.71 (s, 3H), 5.31 (s, 2H), 6.50 (dd, 2H, *J* = 8.4 Hz, 2.0 Hz), 6.58 (d, 1H, *J* = 8.0 Hz), 6.64 (d, 2H, *J* = 8.4 Hz), 6.94 (d, 2H, *J* = 2.0 Hz), 7.75 (d, 1H, *J* = 8.0 Hz), 7.92 (s, 1H). HRMS (ESI$^+$): Calcd for $[M]^+$, 387.17087, Found, 387.16793 (-2.94mmu)

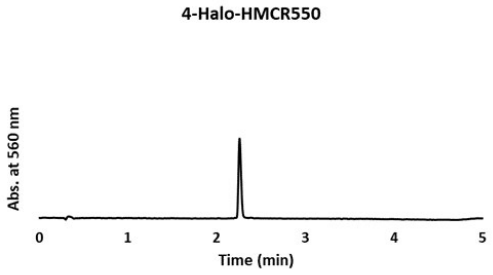
Compound 27 (5-COOH-HMCR550)

To a flame-dried flask flushed with Ar, compound 16 (1630 mg, 4.2 mmol) and anhydrous THF (20 mL) were added. The mixture was cooled to -78˚C and then 1 M *sec*-BuLi (4.2 mL, 4.2 mmol) was added to it. Compound 25 (248 mg, 0.6 mmol) in anhydrous THF (5 mL) was further added. The mixture was stirred at r.t. for 6 hours, then the reaction was quenched with 2 N HCl aq., and the mixture was extracted with AcOEt from saturated $NaHCO_3$ aq.. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was dissolved in methanol (5 mL), and the solution was stirred at 0˚C. Sodium tetrahydroborate (68 mg, 1.8 mmol) was added and stirring was continued at r.t. for 10 minutes. The reaction was quenched with H_2O and the mixture was extracted with AcOEt. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was dissolved in dehydrated CH_2Cl_2 (20 mL), and 1,3-dimethylbarbituric acid (469 mg, 3.0 mmol) and $Pd(PPh_3)_4$ (139 mg, 0.2 mmol) were added. The resulting solution was stirred at 40˚C under an Ar atmosphere for 24 hours. The mixture was evaporated and dissolved in CH_2Cl_2 (5 mL). Then chloranil (192 mg, 0.78 mmol) and TFA (5 mL) were added, and stirring was continued at r.t. for 10 minutes. The mixture was evaporated. The residue was purified by preparative HPLC under the following conditions: A/B = 90/10 (0 min) to 0/100 (30 min) linear gradient (solvent A: H_2O, 0.1% TFA; solvent B: acetonitrile/H_2O = 80/20, 0.1% TFA). Compound 27 was obtained as a violet solid (20 mg, 9%). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 7.75 (d, 1H, *J* = 7.8 Hz), 7.21 (d, 1H, *J* = 7.8 Hz), 7.08 (s, 1H), 6.72 (d, 2H, *J* = 2.3 Hz), 6.48 (d, 2H, *J* = 8.2 Hz), 6.31 (dd, 2H, *J* = 8.5, 2.1), 5.17 (s, 2H), 1.62 (s, 3H), 1.53 (s, 3H) ^{13}C-NMR (400 MHz, DMSO-d_6) δ: 148.3, 148.1, 129.8, 128.7, 126.9, 124.5, 120.1, 119.2, 116.2, 113.7, 110.5, 87.6, 72.1, 46.1, 37.4, 34.8, 34.4. HRMS (ESI$^+$): Calcd for $[M+H]^+$, 387.17087, Found, 387.17051 (-0.36 mmu)

Compound 28 was prepared according to the literature[11].

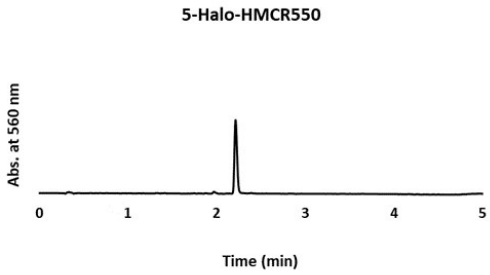
Compound 29 (4-Halo-HMCR550)

Compound 26 (20 mg, 0.05 mmol), compound 28 (33 mg, 0.15 mmol), HOBt (8 mg, 0.05 mmol) and WSCD-HCl (10 mg, 0.05 mmol) were dissolved in anhydrous DMF (2 mL), and the solution was stirred at 0˚C. Triethylamine (15 mg, 0.15 mmol) was added, and stirring was continued at r.t. for 6 hours, then the mixture was extracted with CH_2Cl_2. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by preparative HPLC under the following conditions: A/B = 80/20 (0 min) to 0/100 (30 min) linear gradient (solvent A: H2O, 0.1% TFA; solvent B: acetonitrile/H2O = 80/20, 0.1% TFA). Compound 29 was obtained as an orange solid (2 mg, 6%). ^{1}H NMR (400 MHz, CD_3OD): δ 8.21 (d, J = 1.8 Hz, 1H), 7.94 (dd, J = 7.9, 1.8 Hz, 1H), 7.31 (d, J = 7.9 Hz, 1H), 7.17 (d, J = 2.2 Hz, 2H), 6.99 (d, J = 9.1 Hz, 2H), 6.61 (dd, J = 9.1, 2.2 Hz, 2H), 4.37 (s, 2H), 3.75 – 3.46 (m, 12H), 1.75 (s, 3H), 1.72 (s, 3H), 1.75 – 1.36 (m, 8H). HRMS (ESI$^+$): Calcd for $[M]^+(Cl^{35})$, 592.29421, Found, 592.29497 (+0.77mmu).



Compound 30 (5-Halo-HMCR550)

Compound 27 (9.0 mg, 0.023 mmol), compound 28 (5.2 mg, 0.023 mmol) , HOBt (3.6 mg, 0.023 mmol) and WSCD-HCl (4.4 mg, 0.023 mmol) were dissolved in anhydrous DMF (2 mL), and the solution was stirred at 0˚C. Triethylamine (7.3 mg, 0.073 mmol) was added, and stirring was continued at r.t. for 12 hours, then the mixture was extracted with CH_2Cl_2. The organic solution was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by preparative HPLC under the following conditions: A/B = 90/10 (0 min) to 0/100 (50 min) linear gradient (solvent A: H2O, 0.1% TFA; solvent B: acetonitrile/H2O = 80/20, 0.1% TFA). Compound 30 was obtained as an orange solid (2.1 mg, 13%). ^{1}H NMR (400 MHz, CD_3OD): δ 1.29-1.50 (m, 6H), 1.67 (s, 3H), 1.77 (s, 3H), 1.66-1.76 (m, 4H), 3.36-3.55 (m, 12H), 5.35 (s, 2H), 6.51 (dd, 2H, *J* = 8.4 Hz, 2.4 Hz), 6.63 (d, 2H, *J* = 8.4 Hz), 6.96 (d, 2H, *J* = 2.4 Hz), 7.14 (d, 1H, *J* = 1.4 Hz), 7.49 (d, 1H, *J* = 7.6 Hz), 7.81 (dd, 1H, J = 7.6 , 1.4 Hz). HRMS (ESI$^+$): Calcd for $[M+H]^+$, 592.29421, Found, 592.29554 (1.33 mmu).


5-Halo-HMCR550
Abs. at 560 nm
0
1
2
3
4
5
Time (min)

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