Monochromophoric Iridium(III) Pyridyl-tetrazine Complexes as a Unique Design Strategy for Bioorthogonal Probes with Luminogenic Behavior

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Electronic Supplementary Information

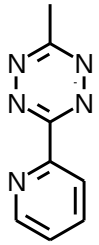
Experimental

Materials and Synthesis

All solvents were of analytical reagent grade and purified according to published procedures.[1] All buffer components were of biological grade and used as received. Autoclaved Milli-Q water was used for the preparation for the aqueous solutions. Hdfppy, Hppy, Hpq, 2-cyanopyridine, hydrazine monohydrate, zinc(II) trifluoromethanesulfonate ($Zn(OTf)_2$), $NaNO_2$, $IrCl_3{\cdot}nH_2O$, $AgNO_3$, 2-(2-*tert*-butoxycarbonylaminoethoxy)ethanol, 6-chloro-1-iodohexane, 1-iodohexane, NaH (60% dispersion in mineral oil), Et_3N, BCN-OH, and (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl-*N*-succinimidyl carbonate (BCN-NHS) were purchased from Sigma Aldrich. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was supplied by Sigma-Aldrich. BSA was purchased from Calbiochem and used as received. The HaloTag standard protein GST-HaloTag and pHTN HaloTag CMV-neo vector were purchased from Promega. PD-10 size-exclusion columns and YM-50 microcon filters were purchased from GE Healthcare and Millipore, respectively. Novex sharp pre-stained protein standard, Dulbecco's modified Eagle's medium (DMEM), Opti-MEM (reduced serum medium), fetal bovine serum (FBS), PBS at pH 7.4, trypsin-EDTA, and penicillin/streptomycin were purchased from Invitrogen. Unless otherwise specified, the growth medium for cell culture contained DMEM with 10% FBS and 1% penicillin/streptomycin. HeLa cells were obtained from American Type Culture Collection. The iridium(III) dimers $[Ir_2(N\text{^}C)_4Cl_2]$ (HN^C = Hdfppy, Hppy, and Hpq)[2] and 1-(*N*-(((1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-yl)methoxycarbonyl)

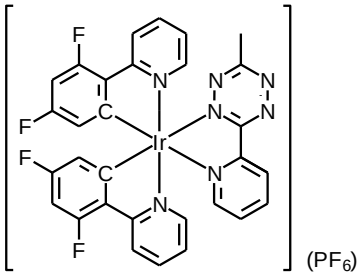
amino)-12-chloro-(3,6-dioxa)-dodecane (BCN-C6-Cl)[3] were prepared by reported methods.

Py-Tz-Me



A mixture of 2-cyanopyridine (400 mg, 3.84 mmol), CH_3CN (2 mL, 39.25 mmol), $Zn(OTf)_2$ (350 mg, 0.96 mmol), and hydrazine monohydrate (4.6 mL, 95 mmol) was heated in an oil bath at 70°C under an inert atmosphere of nitrogen for 72 h. After the mixture was cooled to room temperature, $NaNO_2$ (5.2 g, 76 mmol) in 5 mL H_2O was added, which was followed by the addition of 1 M HCl until pH = 3. The aqueous phase was extracted with CH_2Cl_2 (30 mL × 3). The combined organic phase was dried over $MgSO_4$ and evaporated to dryness yielding a purple solid, which was purified by column chromatography on silica gel. The desired product was eluted with CH_2Cl_2/acetone (20:1, *v*/*v*). The solvent was removed under vacuum to afford the product as a purple solid. Yield: 267 mg (40%). ^1H NMR (400 MHz, $CDCl_3$, 298 K): δ 8.96 (d, 1H, J = 4.0 Hz; H6 of pyridyl ring), 8.64 (d, 1H, J = 8.0 Hz; H3 of pyridyl ring), 7.99 (t, 1H, J = 7.8 Hz; H4 of pyridyl ring), 7.56 (t, 1H, J = 5.6 Hz; H5 of pyridyl ring), 3.16 (s, 3H; CH_3); MS (ESI, positive-ion mode): *m/z* 174.2 $[M + H^+]^+$.

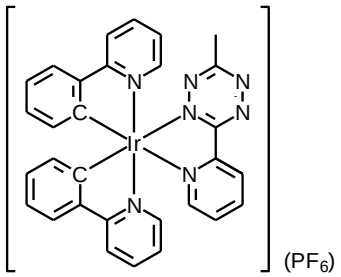
[Ir(dfppy)$_2$(py-Tz-Me)](PF$_6$) (**1**)



A mixture of [Ir$_2$(dfppy)$_4$Cl$_2$] (50 mg, 0.041 mmol) and AgNO$_3$ (28 mg, 0.164 mmol) in EtOH (10 mL) was stirred at room temperature in the dark for 12 h. The solution was filtered and evaporated to dryness under vacuum, leaving a yellow solid. The solid and py-Tz-Me (16 mg, 0.091 mmol) were dissolved in CH_2Cl_2 (10 mL), and the solution was stirred at room temperature in the dark for 12 h. A methanolic solution (1 mL) of KPF$_6$ (38 mg, 0.205 mmol) was added to the mixture and further stirred for 20 min. The mixture was evaporated to dryness yielding a reddish brown residue, which was purified by column chromatography on silica gel. The desired product was eluted with CH_2Cl_2/MeOH (20:1, *v/v*). The solvent was removed under vacuum to yield a reddish brown solid. Subsequent recrystallization from CH_2Cl_2/Et_2O afforded complex **1** as reddish brown crystals. Yield: 31 mg (41%, 0.033 mmol; [Ir(dfppy)$_2$(py-Tz-Me)](PF$_6$)·2H$_2$O). ^{1}H NMR (300 MHz, CD_3COCD_3, 298 K): δ 9.25 (d, 1H, J = 7.8 Hz; H3 of pyridyl ring of py-Tz-Me), 8.57 (t, 1H, J = 7.1 Hz; H4 of pyridyl ring of py-Tz-Me), 8.45 – 8.35 (m, 3H; H6 of pyridyl ring of dfppy and H6 of pyridyl ring of py-Tz-Me), 8.24 (d, 1H, J = 5.4 Hz; H3 of pyridyl ring of dfppy), 8.15 – 8.05 (m, 3H; H5 of pyridyl ring of dfppy and H5 of pyridyl ring of py-Tz-Me), 7.92 (d, 1H, J = 5.4 Hz; H3 of pyridyl ring of dfppy), 7.30 – 7.20 (m, 2H; H4 of pyridyl ring of dfppy), 6.88 – 6.72 (m, 2H; H6 of phenyl ring of dfppy), 5.78 – 5.72 (m, 2H; H4 of phenyl ring of dfppy), 3.02 (s, 3H, CH_3); IR (KBr) ν/cm^{-1}: 841 (PF_6^-);

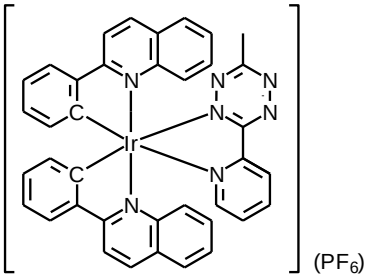
MS (ESI, positive-ion mode): *m/z* 746 $[M - PF_6^-]^+$; elemental analysis calcd (%) for $IrC_{30}H_{19}N_7PF_{10}\cdot 2H_2O$: C, 38.88; H, 2.50; N, 10.58; found: C, 39.00; H, 2.77; N, 10.64.

$[Ir(ppy)_2(py\text{-}Tz\text{-}Me)](PF_6)$ (**2**)



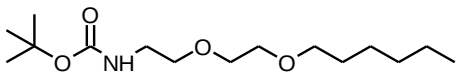
The synthetic procedure was similar to that for complex **1**, except that $[Ir_2(ppy)_4Cl_2]$ (100 mg, 0.093 mmol) was used instead of $[Ir_2(dfppy)_4Cl_2]$. Subsequent recrystallization from CH_2Cl_2/Et_2O afforded complex **2** as brown crystals. Yield: 47 mg (30%, 0.055 mmol; $[Ir(ppy)_2(py\text{-}Tz\text{-}Me)](PF_6)\cdot 2H_2O$). ^1H NMR (300 MHz, CD_3COCD_3, 298 K): δ 9.20 (d, 1H, J = 7.8 Hz; H3 of pyridyl ring of py-Tz-Me), 8.50 (t, 1H, J = 7.5 Hz; H4 of pyridyl ring of py-Tz-Me), 8.35 – 8.15 (m, 4H; H3 and H4 of pyridyl ring of ppy), 8.05 – 7.95 (m, 4H; H5 and H6 of pyridyl ring of ppy), 7.90 – 7.80 (m, 2H; H3 of phenyl ring of ppy), 7.20 – 7.05 (m, 3H; H4 of phenyl ring of ppy and H6 of pyridyl ring of py-Tz-Me), 7.00 (t, 2H, J = 7.5 Hz; H5 of phenyl ring of ppy), 6.90 (t, 1H, J = 7.5 Hz; H5 of pyridyl ring of py-Tz-Me), 6.26 (d, 2H, J = 7.5 Hz; H6 of phenyl ring of ppy), 2.97 (s, 3H, CH_3); IR (KBr) ν/cm^{-1}: 841 (PF_6^-); MS (ESI, positive-ion mode): *m/z* 674 $[M - PF_6^-]^+$; elemental analysis calcd (%) for $IrC_{30}H_{23}N_7PF_6\cdot 2H_2O$: C, 42.16; H, 3.18; N, 11.47; found: C, 42.33; H, 3.26; N, 11.50.

$[Ir(pq)_2(py\text{-}Tz\text{-}Me)](PF_6)$ (**3**)



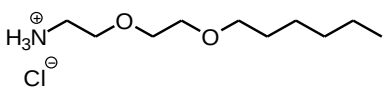
The synthetic procedure was similar to that for complex **1**, except that $[Ir_2(pq)_4Cl_2]$ (40 mg, 0.032 mmol) was used instead of $[Ir_2(dfppy)_4Cl_2]$. Subsequent recrystallization from CH_2Cl_2/Et_2O afforded complex **3** as brown crystals. Yield: 32 mg (52%, 0.034 mmol; $[Ir(pq)_2(py\text{-}Tz\text{-}Me)](PF_6)\cdot 2H_2O$). ^1H NMR (400 MHz, $CDCl_3$, 298 K): δ 8.55 (d, 1H, J = 8.0 Hz; H8 of quinolinyl ring of pq), 8.40 (d, 1H, J = 5.6 Hz; H8 of quinolinyl ring of pq), 8.35 – 8.25 (m, 4H; H3 and H4 of quinolinyl ring of pq), 8.18 – 8.11 (m, 2H; H3 of phenyl ring of pq and H3 of pyridyl ring of py-Tz-Me), 8.04 (d, 1H, J = 8.0 Hz; H3 of phenyl ring of pq), 7.92 (t, 1H, J = 6.0 Hz; H4 of pyridyl ring of py-Tz-Me), 7.79 – 7.73 (m, 2H; H5 of quinolinyl ring of pq), 7.41 – 7.31 (m, 2H; H6 of quinolinyl ring of pq), 7.21 (t, 1H, J = 7.6 Hz; H4 of phenyl ring of pq), 7.18 – 7.10 (m, 2H; H4 of phenyl ring of pq and H6 of pyridyl ring of py-Tz-Me), 7.09 – 7.00 (m, 3H; H7 of quinolinyl ring of pq and H5 of pyridyl ring of py-Tz-Me), 6.92 (t, 1H, J = 7.6 Hz; H5 of phenyl ring of pq), 6.86 (t, 1H, J = 7.6 Hz; H5 of phenyl ring of pq), 6.60 (d, 1H, J = 7.6 Hz; H6 of phenyl ring of pq), 6.48 (d, 1H, J = 7.6 Hz; H6 of phenyl ring of pq), 2.99 (s, 3H; CH_3); IR (KBr) ν/cm^{-1}: 843 (PF_6^-); MS (ESI, positive-ion mode): m/z 774 $[M - PF_6^-]^+$; elemental analysis calcd (%) for $IrC_{38}H_{27}N_7PF_6\cdot 2H_2O$: C, 47.80; H, 3.27; N, 10.27; found: C, 48.03; H, 3.65; N, 10.51.

1-(*N*-(*tert*-butoxycarbonyl)amino)-3,6-dioxa-dodecane (C6-Boc)



A mixture of 2-(2-*tert*-butoxycarbonylaminoethoxy)ethanol (188.5 μL, 0.97 mmol) and NaH (60% dispersion in mineral oil, 50 mg, 1.25 mmol) in THF/DMF (10 mL, 9:1, *v/v*) was stirred at 0°C for 45 min. Then, 1-iodohexane (215 μL, 1.46 mmol) was added to the mixture and it was further stirred at room temperature for 24 h. A saturated aqueous solution of NH_4Cl was added to the quench the reaction mixture. The aqueous phase was extracted with EtOAc (30 mL × 3). The combined organic phase was dried over $MgSO_4$ and evaporated to dryness under vacuum yielding a colorless oil, which was purified by column chromatography on silica gel. The desired product was eluted with hexane/EtOAc (5:1, *v/v*). The solvent was removed under vacuum to afford the product as a colorless oil. Yield: 85 mg (30%). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ 5.07 (br s, 1H; CONH), 3.63 – 3.52 (m, 6H; $NCH_2CH_2OCH_2CH_2O$), 3.45 (t, 2H, J = 6.8 Hz; $OCH_2CH_2CH_2$), 3.34 – 3.28 (m, 2H; NCH_2), 1.63 – 1.54 (m, 2H; $OCH_2CH_2CH_2$), 1.43 (s, 9H; Boc), 1.38 – 1.23 (m, 6H; $CH_2CH_2CH_2CH_3$), 0.90 – 0.85 (m, 3H; CH_3); MS (ESI, positive-ion mode): *m/z* 290.5 $[M + H^+]^+$.

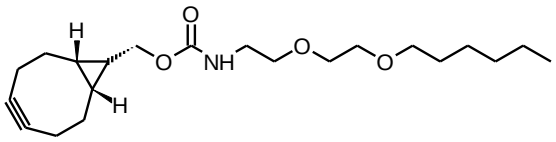
1-(3,6-dioxa-dodecyl)ammonium chloride (C6-$NH_3^+Cl^-$)



A mixture of C6-Boc (85 mg, 0.29 mmol) and HCl (12 M, 3 mL) in MeOH (9 mL)

was stirred at room temperature for 12 h. The solution was neutralized by NH_4OH solution and then concentrated under vacuum. The aqueous phase was extracted with CH_2Cl_2 (20 mL × 3). The combined organic phase was dried over $MgSO_4$ and evaporated to dryness under vacuum yielding a colorless oil, which was purified by column chromatography on silica gel. The desired product was eluted with CH_2Cl_2/MeOH (5:1, *v/v*). The solvent was removed under vacuum to afford the product as a colorless oil. Yield: 40 mg (61%). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ 3.86 – 3.80 (m, 2H; NCH_2CH_2O), 3.70 – 3.60 (m, 4H; OCH_2CH_2O), 3.47 (t, 2H, J = 6.8 Hz; $OCH_2CH_2CH_2$), 3.26 (br s, 5H; $^+H_3NCH_2$), 1.63 – 1.55 (m, 2H; $OCH_2CH_2CH_2$), 1.35 – 1.25 (m, 6H; $CH_2CH_2CH_2CH_3$), 0.95 – 0.85 (m, 3H; CH_3); MS (ESI, positive-ion mode): *m/z* 190.6 $[M - Cl^-]^+$.

1-(*N*-(((1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-yl)methoxycarbonyl)amino)-(3,6-dioxa)-dodecane (BCN-C6)



A mixture of C6-$NH_3^+Cl^-$ (20.5 mg, 0.091 mmol), BCN-NHS (13.1 mg, 0.045 mmol), and Et_3N (38 μL, 0.27 mmol) in anhydrous CH_2Cl_2 (5 mL) was stirred at room temperature for 12 h. The solution was extracted with H_2O (10 mL). The organic phase was dried over $MgSO_4$ and evaporated to dryness under vacuum yielding a colorless oil, which was purified by column chromatography on silica gel. The desired product was eluted with CH_2Cl_2/MeOH (20:1, *v/v*). The solvent was removed under vacuum to afford the product as a colorless oil. Yield: 11 mg

(67%). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ 5.25 (br s, 1H; CONH), 4.16 (d, 2H, J = 8.0 Hz; O-CH_2-BCN), 3.65 – 3.55 (m, 6H; $NCH_2CH_2OCH_2CH_2O$), 3.47 (t, 2H, J = 6.8 Hz; $OCH_2CH_2CH_2$), 3.42 – 3.36 (m, 2H; NCH_2), 2.28 – 2.22 (m, 4H; BCN), 1.75 (s, 1H; BCN), 1.65 – 1.55 (m, 4H; $OCH_2CH_2CH_2$ and BCN), 1.40 – 1.26 (m, 8H; $CH_2CH_2CH_2CH_3$ and BCN), 1.00 – 0.87 (m, 5H; CH_3 and BCN); MS (ESI, positive-ion mode): m/z 366.3 $[M + H^+]^+$.

Instrumentation and Methods

The instruments for the characterization and photophysical measurements have been described previously.[4] Emission quantum yields were measured using the optically dilute method[5] with an aerated aqueous solution of $[Ru(bpy)_3]Cl_2$ (Φ_{em} = 0.028) as the standard solution.[6]

Kinetics Studies

The IEDDA reaction kinetics of the tetrazine complexes (5 μM) with BCN-OH (40 – 250 μM) in CH_3CN/H_2O (1:1, *v/v*) at 298 K were measured by emission spectroscopy. The reactions were monitored by following the increase in the emission maxima of the complexes upon addition of BCN-OH. Also, the reaction of the free ligand py-Tz-Me (5 μM) with BCN-OH (100 – 250 μM) in CH_3CN/H_2O (1:1, *v/v*) at 298 K was conducted by UV/VIS absorption spectroscopy and monitored by following the decrease in the absorbance of the ligand at 273 nm. Data were fitted to a single-exponential equation to give the pseudo-first-order rate constants k_{obs}, which were then plotted against the concentrations of BCN-OH to obtain the second-order rate constant k_2.

Stability toward Biothiols

Complex **3** (25 μM) was incubated with a biothiol (cysteine or glutathione, 1 mM), in aerated 50% aqueous MeOH for 3 and 6 h. Without further purification, the mixtures were subject to ESI-MS analysis to determine the stability of the complex toward the biothiols.

Single-step Labeling of BCN-modified BSA

Modification of BSA with BCN-NHS. BCN-NHS (1.32 mg, 4.55 μmol) in 100 μL anhydrous DMSO was added to BSA (30 mg, 0.47 μmol) dissolved in 400 μL carbonate buffer (50 mM, pH 10). The mixture was incubated in the dark at 298 K for 24 h. The solution was loaded onto a PD-10 size exclusion column that had been preequilibrated with potassium phosphate buffer (50 mM, pH 7.4). Volume fractions between 2.5 and 5.0 mL were collected, washed with the same buffer with a YM-50 microcon filter, and finally concentrated to 1.0 mL and stored at 4°C. Positive-ion MALDI-TOF MS ion cluster at *m/z* 66,730 $[M + H]^+$ indicated that an average BSA molecule was modified with *ca.* one BCN unit (Fig. S8).

Labeling of BCN-BSA with the tetrazine complexes. The complex (2.5 nmol) in 5 μL anhydrous DMSO was added to BCN-BSA or unmodified BSA (M_W = *ca.* 66 kDa) (1 nmol) in 495 μL potassium phosphate buffer (50 mM, pH 7.4). The mixture was incubated in the dark at 298 K for 2 h. The final concentration of the protein and complex in potassium phosphate buffer (50 mM, pH 7.4) containing 1% DMSO was 2 and 5 μM, respectively. The emission spectrum of the solution was recorded, and an aliquot of the mixture was analyzed by SDS-PAGE and MALDI-TOF analyses. Positive-ion MALDI-TOF MS ion clusters at *m/z* 67,471 [**1**–BSA]$^+$, 67,320 [**2**–BSA]$^+$, and 67,389 [**3**–BSA]$^+$ indicated that the [Ir]:[BSA] ratios of these iridium

complex-BSA conjugates were *ca.* 1.0 (Fig. S10–S12).

Two-step Labeling of the GST-HaloTag fusion protein

BCN-C6-Cl (24.6 pmol) in 3.5 μL anhydrous DMSO was added to the GST-HaloTag fusion protein (M_W = *ca.* 62 kDa) (24.6 pmol) dissolved in 63 μL PBS. The mixture was incubated in the dark at 298 K for 1 h. Complex **3** (24.6 pmol) in 3.5 μL anhydrous DMSO was added to the mixture, which was further incubated in the dark at 298 K for 2 h. The final concentration of the protein, BCN-C6-Cl, and complex **3** in PBS/DMSO (70 μL, 9:1, *v/v*) was 350 nM. The emission spectrum of the solution was recorded. Also, an aliquot of the mixture was analyzed by SDS-PAGE. For the control experiments, BCN-C6 was incubated with the protein and then treated with the complex. Additionally, complex **3** (24.6 pmol) was incubated with BCN-C6-Cl or BCN-C6 (24.6 pmol) in PBS/DMSO (70 μL, 9:1, *v/v*) for 2 h, and the reaction mixtures were diluted with MeOH (1 mL) and then subject to ESI-MS analysis to confirm the formation of the pyridazine derivatives (Scheme 3 and Fig. S13).

Lipophilicity

The lipophilicity (log $P_{o/w}$ values) of the tetrazine complexes was measured using the shake-flask method. An aliquot of stock solution of the complex in *n*-octanol (saturated with aqueous NaCl solution) was added to an equal volume of aqueous NaCl solution (0.9%, *w/v*, saturated with *n*-octanol). The mixture was shaken vigorously for 4 h and then centrifuged for 30 min for phase separation. The two immiscible layers were carefully isolated and the concentration of the complex in the organic layer was determined by electronic absorption spectroscopy.

Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES)

HeLa cells were seeded in a 60-mm tissue culture dish and incubated in medium/DMSO (99:1, *v/v*) containing the tetrazine complex (20 μM) at 37°C under a 5% CO_2 atmosphere. After incubation for 2 h, the medium was removed and the cells were washed thoroughly with PBS. The cells were typsinized by trypsin-EDTA (500 μL) and harvested. The culture dish was further washed with PBS (500 μL × 3) and the PBS portions were collected. The harvested cells, together with the collected PBS, were digested with 65% HNO_3 (1 mL) at 60°C. The concentration of iridium in the solution was measured using an Optima 8000 ICP-OES system (PerkinElmer, Inc., USA).

MTT Assays

HeLa cells were seeded in a 96-well flat-bottomed microplate and incubated in medium/DMSO (100 μL, 99:1, *v/v*) containing the tetrazine complex (1.25 – 80 μM) in the dark at 37°C under a 5% CO_2 atmosphere for 2 and 24 h. The cell viability was determined by the MTT assay. The absorbance of the solutions at 570 nm was measured with a SPECTRAmax 340 microplate reader (Molecular Devices Corp., Sunnyvale, CA).

Fixed Cell Confocal imaging

HeLa cells were seeded on a sterilized glass coverslip in a 35-mm tissue culture dish. The culture medium was removed and replaced with cold MeOH (2 mL, −20°C). After incubation for 5 min, the MeOH was removed, the cells were washed with PBS, then incubated in PBS/DMSO (99:1, *v/v*) containing BCN-NHS (200 μM) at room temperature for 2 h, and subsequently washed with PBS. The BCN moiety can be

coupled to any amine-containing molecules in the fixed cells. The BCN-labeled fixed cells were incubated with complex **3** (25 μM) in PBS/DMSO (99:1, *v/v*) at 37°C under a 5% CO_2 atmosphere for 1 h and imaged using a Leica TCS SPE (inverted configuration) confocal microscope and a 63× oil-immersion objective lens.

Preparation of Lipoplexes

The pHTN HaloTag® CMV-neo Vector (1.5 μg), which is a plasmid DNA (pDNA) encoding the HaloTag protein, and the transfection reagent Lipofectamine 2000 (3 μL) were incubated in Opti-MEM (100 μL) individually at 298 K for 5 min. The pDNA and Lipofectamine 2000 solutions were then combined, mixed gently, and incubated at 298 K for 20 min to allow the formation of lipoplexes.

***In Vitro* Transfection**

HeLa cells were seeded in a 35-mm tissue culture dish and incubated at 37°C under a 5% CO_2 atmosphere for 48 h. When the cells were grown at 90% confluency, the medium was replaced with Opti-MEM (2 mL) containing the lipoplexes pDNA/Lipofectamine 2000. After incubation for 4 h, the medium was removed, the cells were washed thoroughly with PBS, and incubated in an antibiotic-free medium (DMEM containing 10% FBS) for 18 h. The transfected cells that express HaloTag protein were then used for live cell imaging.

Live Cell Confocal Imaging

Transfected HeLa cells that express HaloTag protein were seeded on a sterilized glass coverslip in a 35-mm tissue culture dish. They were incubated in antibiotic-free medium/DMSO (99:1, *v/v*) containing BCN-C6-Cl (200 μM) at 37°C under a 5% CO_2

atmosphere for 45 min. The medium was removed, the cells were washed thoroughly with PBS, and then incubated in an antibiotic-free medium containing complex **3** (5 μM) for 2 h. After treatments, the cells were incubated in a fresh antibiotic-free medium for an additional 4 h. After washing with PBS, the cells were imaged using a Leica TCS SPE (inverted configuration) confocal microscope and a 63× oil-immersion objective lens. Control experiments involving nontransfected HeLa cells treated with complex **3** only, and nontransfected cells treated with both BCN-C6-Cl and complex **3** under the same incubation conditions were also carried out.

References

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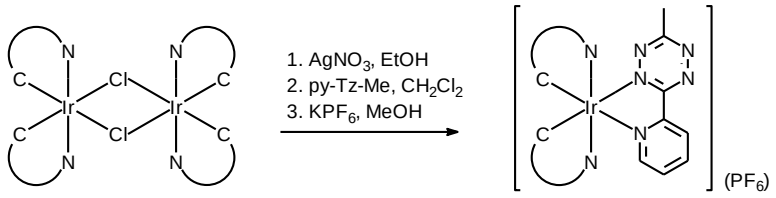
Scheme S1 Synthesis of complexes **1** – **3**.



N^C⁻ = dfppy⁻ (**1**), ppy⁻ (**2**), pq⁻ (**3**)

Table S1 Electronic absorption data of complexes **1** – **3** in MeOH at 298 K

Complex	λ_{abs}/nm (ε/dm^3 mol^{-1} cm^{-1})
1	252 (44,690), 313 sh (20,695), 365 sh (10,575), 538 sh (860)
2	264 (50,970), 309 sh (22,720), 386 sh (9,730), 500 sh (1,510), 640 sh (580)
3	262 sh (61,750), 280 (64,750), 334 (34,850), 351 sh (30,670), 426 sh (8,760), 674 sh (545)

Table S2 Photophysical data of complexes **1** – **3**

Complex	Medium	T/K	λ_{em}/nm	Φ_{em}	τ_o/μs
1	CH_2Cl_2	298	465, 544 (max)	0.0053	0.98
	Buffer[a]	298	456, 488 sh, 550 (max)	0.0023	0.31
	Glass[b]	77	449, 473 (max), 505, 551 sh		
2	CH_2Cl_2	298	490, 516 sh	0.0012	1.28
	Buffer[a]	298	484, 513 (max)	0.0001	1.17
	Glass[b]	77	473, 513 (max)		
3	CH_2Cl_2	298	571	0.0084	0.96
	Buffer[a]	298	592	0.0025	0.24
	Glass[b]	77	503, 546 (max), 585		

[a] Potassium phosphate buffer (50 mM, pH 7.4) containing 30% MeOH.

[b] EtOH/MeOH (4:1, *v/v*).

Table S3 Emission enhancement factors (I/I_o) of complexes **1** – **3** upon incubation with BCN-BSA and unmodified BSA

Complex	I/I_o[a]	
	BCN-BSA	unmodified BSA
1	128.1	1.3
2	538.2	3.9
3	1372.2	49.4

[a] I_o and I are the emission intensities of the complexes (5 μM) in the absence and presence of the proteins (2 μM), respectively, in aerated potassium phosphate buffer (50 mM, pH 7.4) containing 1% DMSO at 298 K; incubation time = 2 h.

Table S4 Lipophilicity, cellular uptake, and cytotoxicity of complexes **1** – **3**

Complex	Log $P_{o/w}$[a]	Amount of iridium[b]/fmol	IC_{50}[c]/μM, 2 h (24 h)
1	1.52 ± 0.10	2.31 ± 0.09	> 40 (16.4 ± 0.4)
2	1.01 ± 0.08	1.02 ± 0.02	> 40 (28.8 ± 0.7)
3	2.27 ± 0.28	1.44 ± 0.09	> 40 (17.6 ± 1.8)

[a] Log $P_{o/w}$ is defined as the logarithmic ratio of the concentration of the complex in *n*-octanol (saturated with NaCl) to that in an aqueous solution of NaCl (0.9%, *w*/*v*, saturated with *n*-octanol). [b] Number of moles of iridium associated with an average HeLa cell upon incubation with the complexes (20 μM) at 37°C for 2 h. [c] Cytotoxicity of the complexes toward HeLa cells upon incubation at 37°C for 2 and 24 h.

Fig. S1 Electronic absorption spectra of complexes **1** – **3** in MeOH at 298 K.

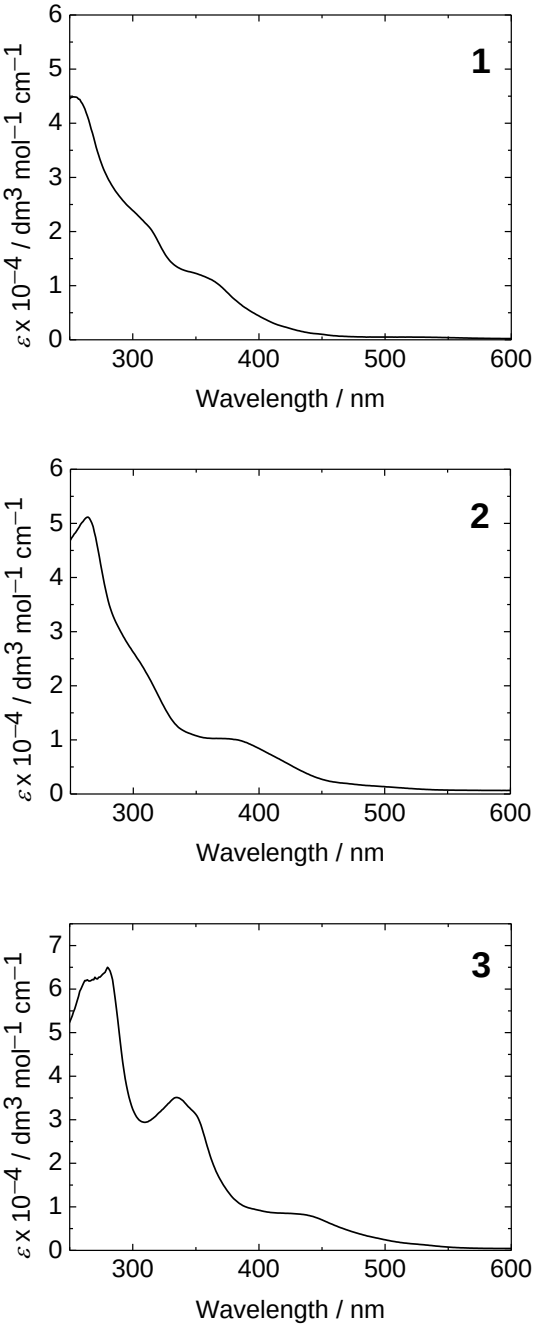


Fig. S2 Emission spectra of complexes **1** – **3** in degassed aqueous buffer at 298 K (solid) and in EtOH/MeOH (4:1, *v/v*) at 77 K (dashed).

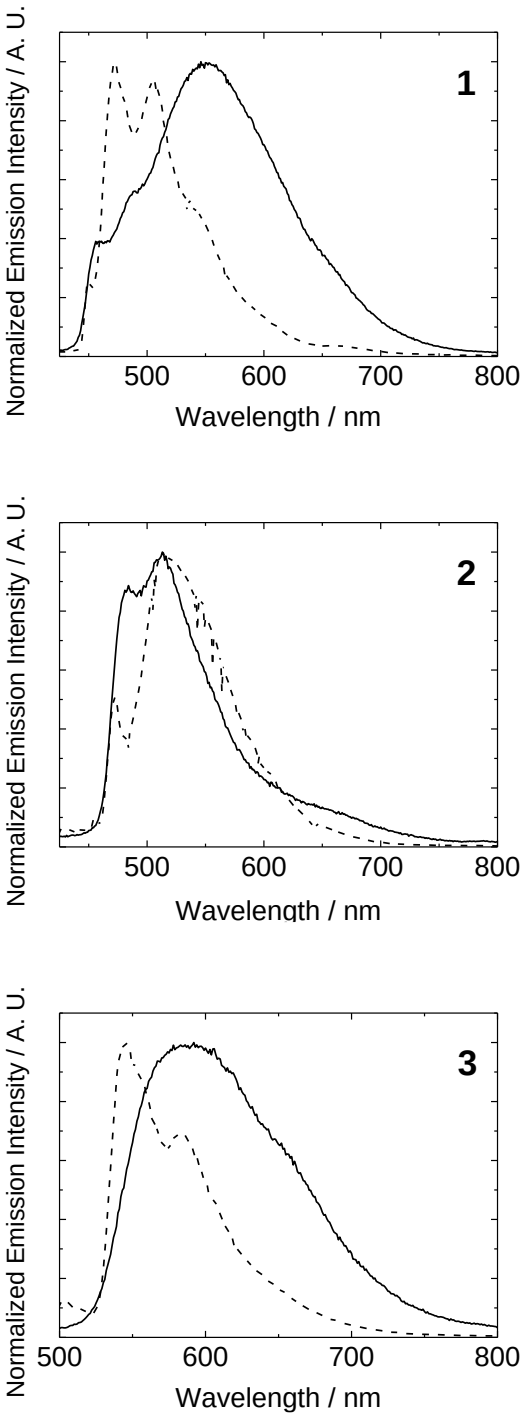


Fig. S3 Emission spectra of complexes **1** – **3** (5 μM) in the absence (blue) and presence (red) of BCN-OH (250 μM) in CH_3CN/H_2O (1:1, *v/v*) at 298 K.

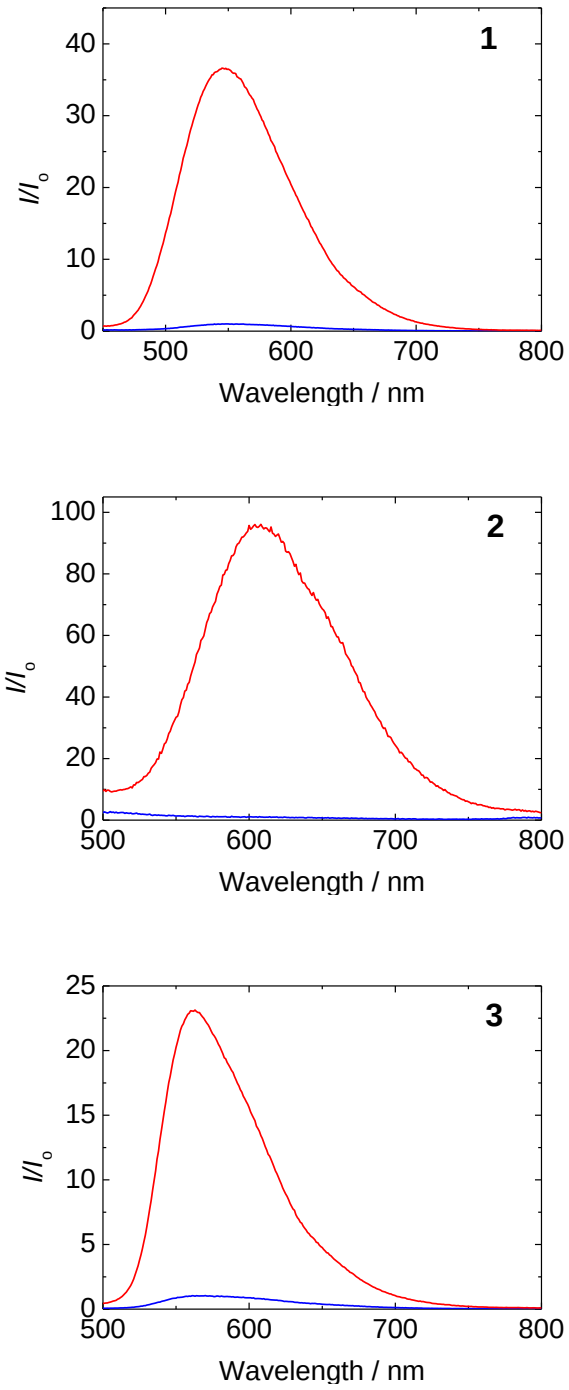


Fig. S4 ESI mass spectra of complexes **1** – **3** and their pyridazine products **1-pz** – **3-pz** formed from the reaction with BCN-OH.

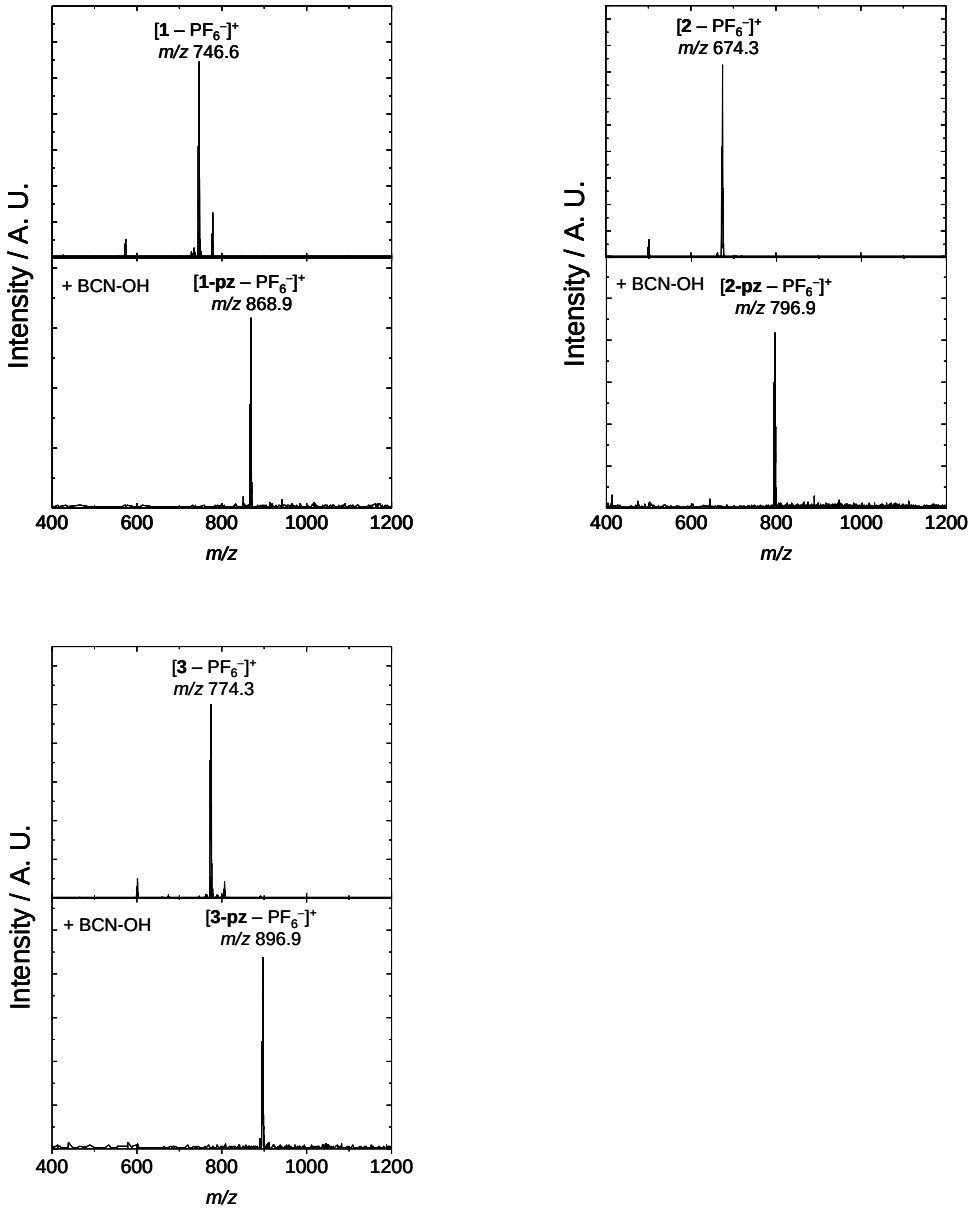


Fig. S5 Normalized changes of emission [$(I - I_{initial})/(I_{final} - I_{initial})$] for the reaction of complexes **1** (top), **2** (middle), and **3** (bottom) with BCN-OH at different concentrations in CH_3CN/H_2O (1:1, *v/v*) at 298 K.

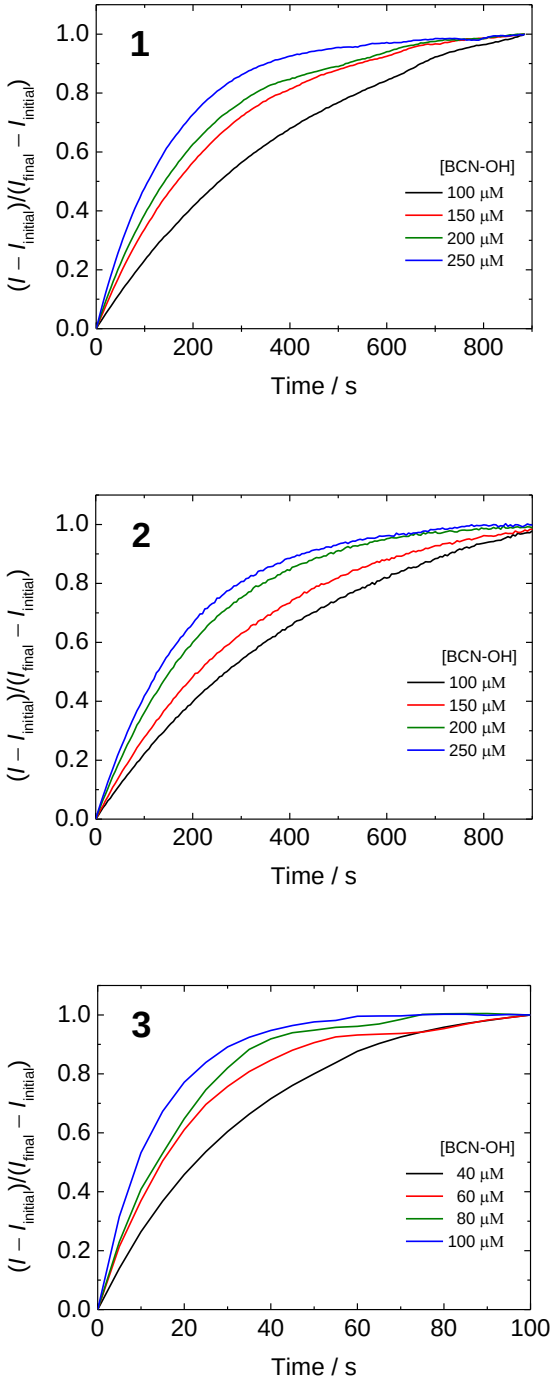


Fig. S6 Normalized changes of absorbance [(A – A_{final})/($A_{initial}$ – A_{final})] for the reaction of the free ligand py-Tz-Me with BCN-OH at different concentrations in CH_3CN/H_2O (1:1, *v/v*) at 298 K.

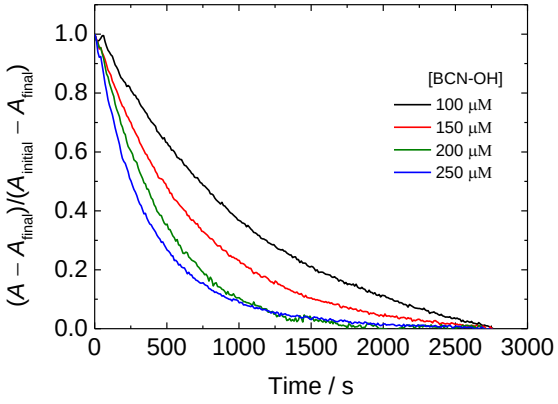


Fig. S7 Pseudo first-order kinetics for the reactions of complexes **1** – **3** and the free ligand py-Tz-Me with BCN-OH at different concentrations in CH_3CN/H_2O (1:1, *v/v*) at 298 K. The slope corresponds to the k_2 of the reaction.

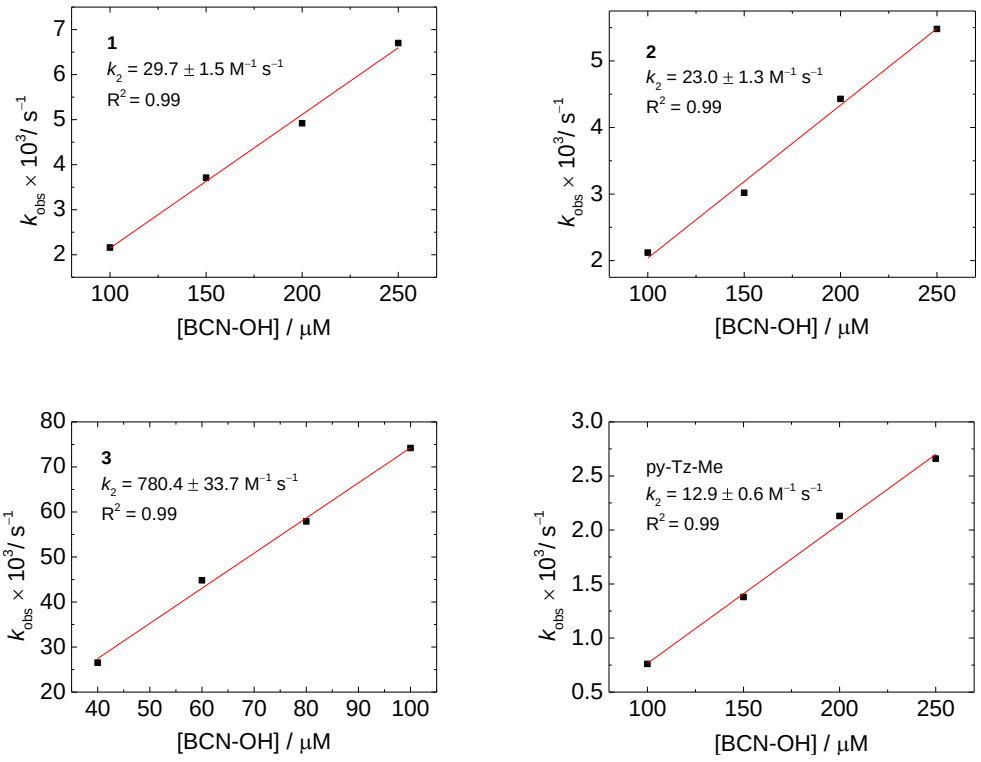


Fig. S8 MALDI-TOF-mass spectra of BSA (black) and BCN-BSA (red).

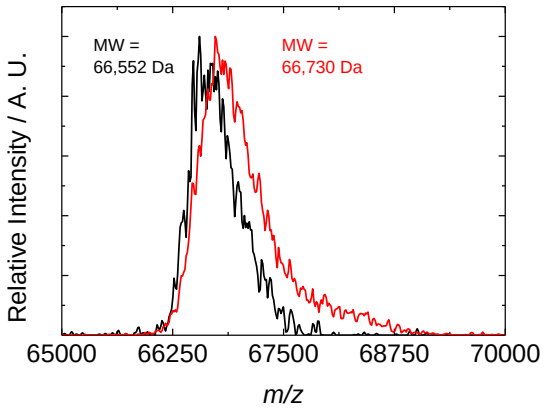


Fig. S9 SDS-PAGE analysis. Left: UV transillumination; right: Coomassie Blue staining. Lane 1: protein ladder; lanes 2, 4, and 6: complexes **1**, **2**, and **3** with unmodified BSA, respectively; lanes 3, 5, and 7: complexes **1**, **2**, and **3** with BCN-BSA, respectively.

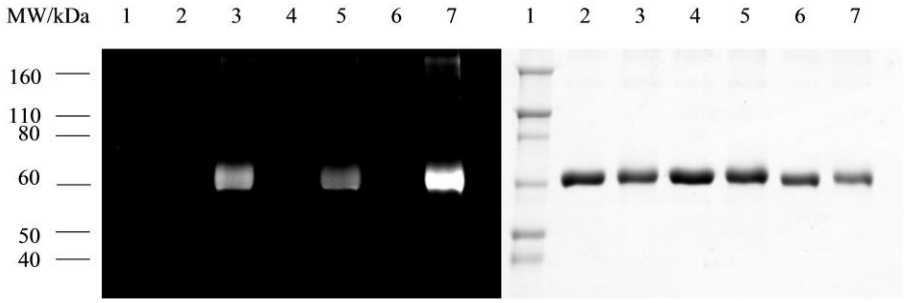


Fig. S10 MALDI-TOF-mass spectra of BCN-BSA (black) and BCN-BSA labeled by complex **1** (red).

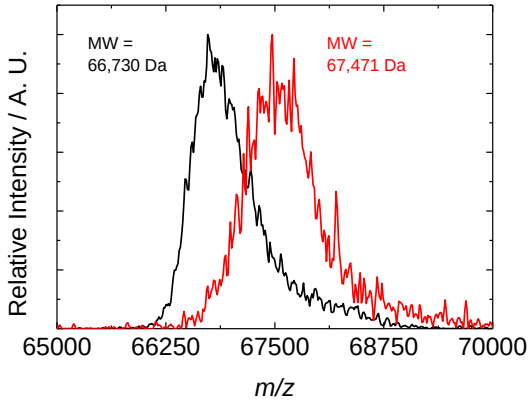


Fig. S11 MALDI-TOF-mass spectra of BCN-BSA (black) and BCN-BSA labeled by complex **2** (red).

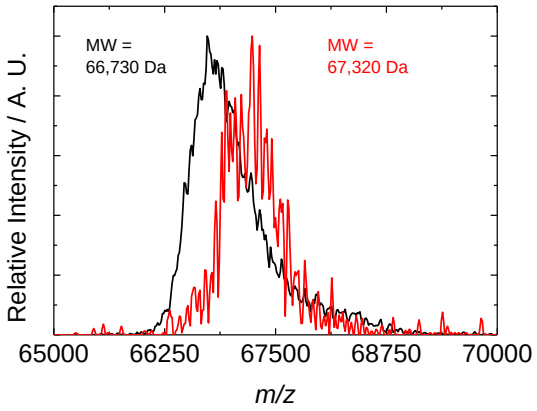


Fig. S12 MALDI-TOF-mass spectra of BCN-BSA (black) and BCN-BSA labeled by complex **3** (red).

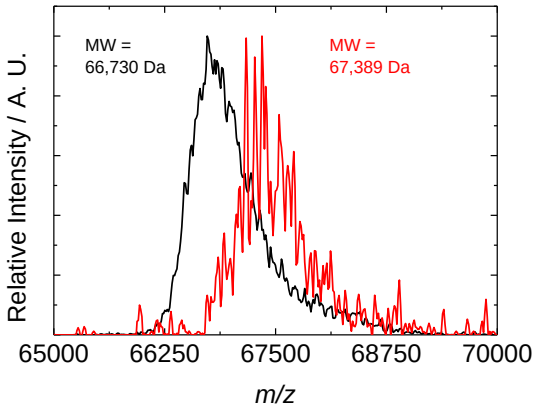
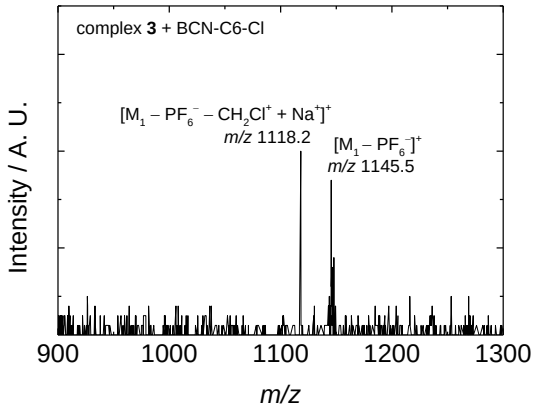


Fig. S13 ESI mass spectra of the reaction mixtures of (a) complex **3** and BCN-C6-Cl and (b) complex **3** and BCN-C6.

(a)



(b)

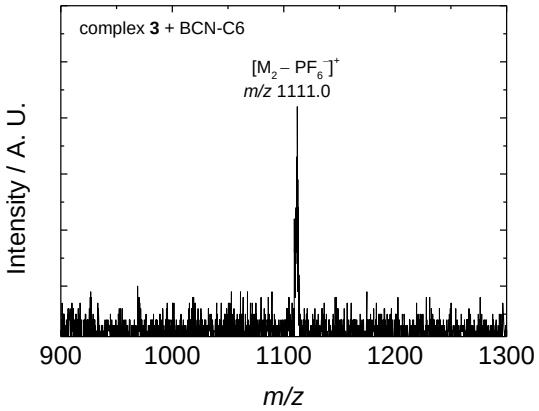


Fig. S14 LSCM images of HeLa cells fixed with MeOH (–20°C, 5 min), then treated with or without BCN-NHS (200 μM) at room temperature for 2 h, washed thoroughly with PBS, and subsequently incubated with complex **3** (25 μM) at 37°C for 1 h.

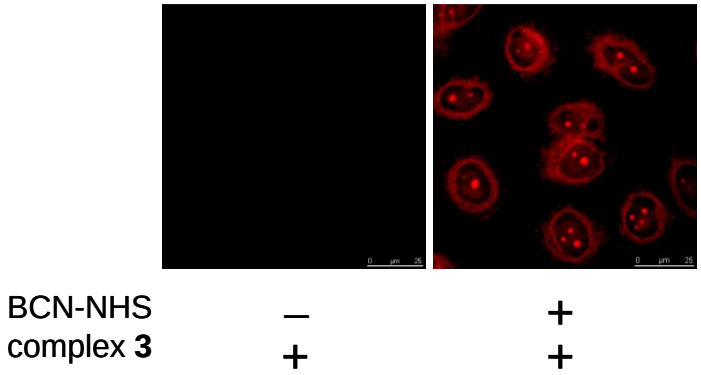


Fig. S15 ESI mass spectra of the mixture of complex **3** (25 μM; *m/z* 774) and cysteine (1 mM) in 50% aqueous MeOH after incubation for 3 (top) and 6 h (bottom).

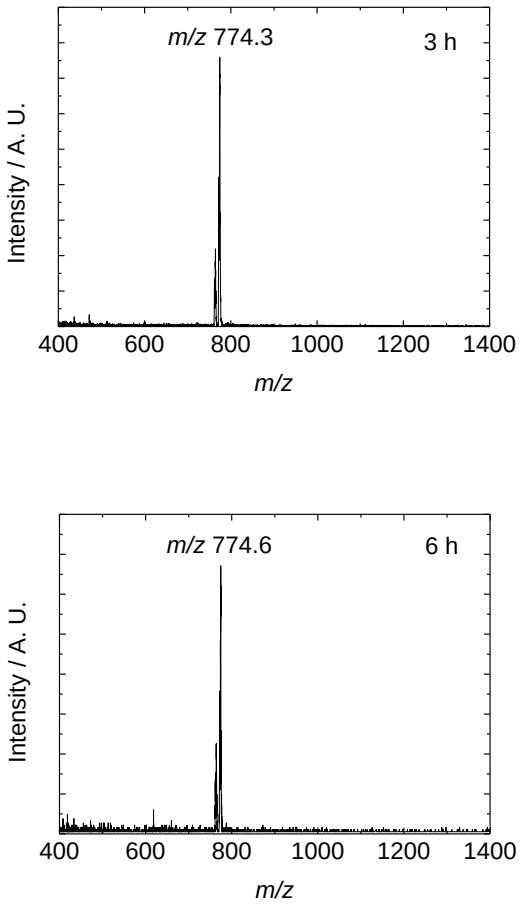


Fig. S16 ESI mass spectra of the mixture of complex **3** (25 μM; *m/z* 774) and glutathione (1 mM) in 50% aqueous MeOH after incubation for 3 (top) and 6 h (bottom).