Development of Cell-Impermeable Coelenterazine Derivatives

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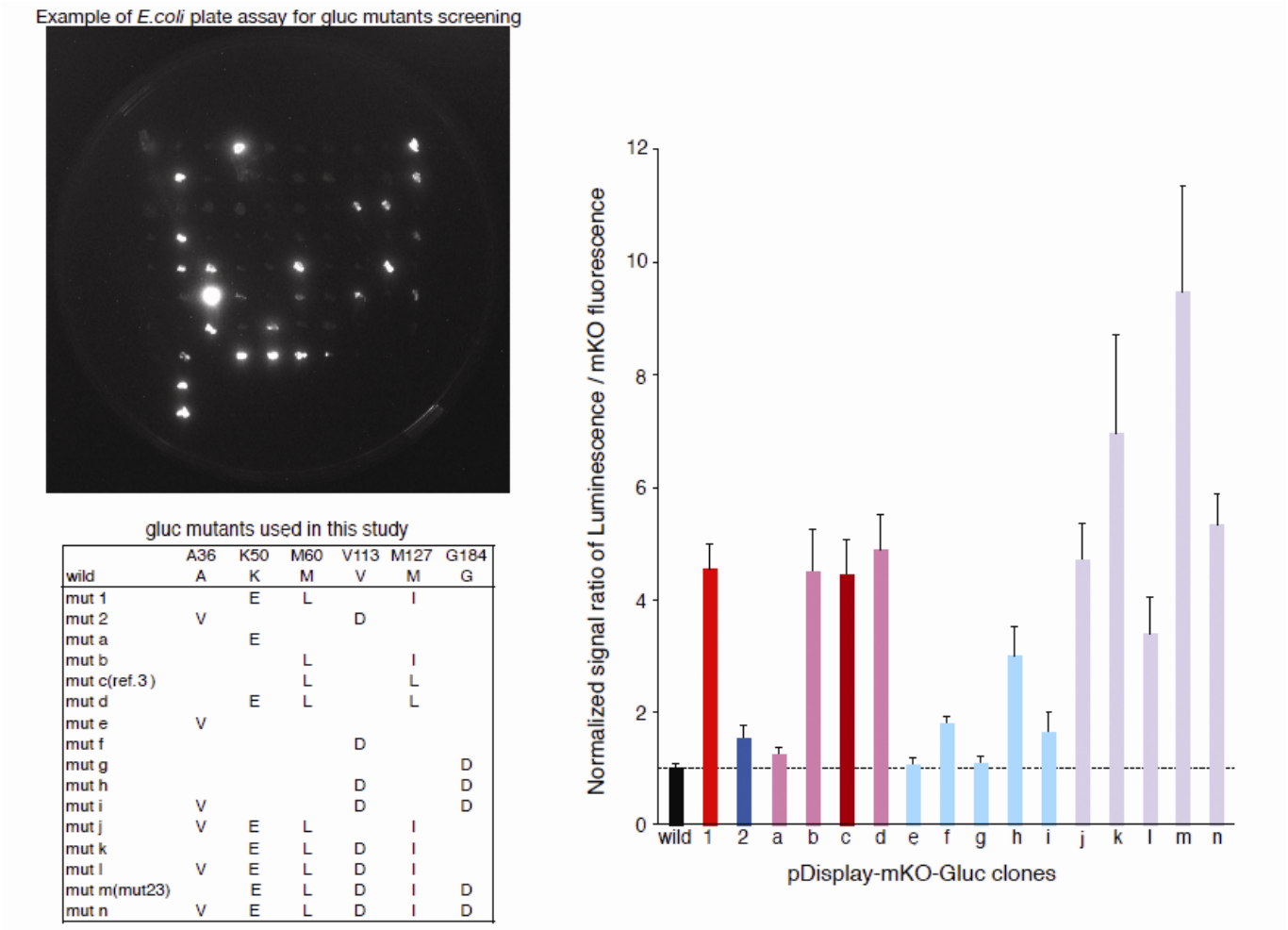
1. Materials and Instruments

General reagents and chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louse, MO), Tokyo Chemical Industries (Tokyo, Japan), and Wako Pure Chemical (Osaka, Japan) and were used without further purification. Silica gel chromatography was performed using BW-300 (Fuji Silisia Chemical Ltd., Greenville, NC). pcDNA4™/TO/myc-His/lacZ was purchased from Life Technologies Corporation (Japan) NMR spectra were recorded on a JEOL JNM-AL400 instrument at 400 MHz for ^{1}H and 100.4 MHz for ^{13}C NMR, using tetramethylsilane as an internal standard. Mass spectra were measured on a Waters LCT-Premier XE mass spectrometer for ESI or on a JEOL JMS-700 for FAB. UV-visible absorbance spectra were measured using a Shimadzu UV1650PC spectrometer. High pressure liquid chromatography (HPLC) analysis was performed with an Inertsil ODS3 column (4.6 × 250 mm, GL Science, Inc. Torrance, CA) using an HPLC system that comprised a pump (PU2080, JASCO) and a detector (MD2010 and FP2020, JASCO). Preparative HPLC was performed with an Inertsil ODS3 column (10.0 × 250 mm)(GL Sciences Inc.) using an HPLC system with a pump (PU-2087, JASCO) and a detector (UV-2075, JASCO). Bioluminescence was measured in 96-optiplate multiwell plates (PerkinElmer Co., Ltd.) using a Wallac ARVO mx / light 1420 Multilabel / Luminescence counter with an auto-injector (PerkinElmer Co., Ltd.). Bioluminescence imaging was performed utilizing an Olympus DP30 Cooled Monochrome CCD Microscope Camera with a 60 × objective lens. Coelenterazine was synthesized as previously described and stored as 10 mM MeOH/HCl (<1%) solution aliquotes in sealed glass ampulles at -80°C.[1,2]

Mutant GLuc

The cDNA for *Gaussia* luciferase (GLuc) was purchased from New England BioLabs Inc. The DNA sequence coding for GLuc without sequence for signal peptide (dspGLuc, aa18-) was amplified by PCR with primers containing the sequence encoding NdeI site, a bacterial periplasm localization signal (pelB) and XhoI site at the 5′ end and PstI site at the 3′ end. The entire cDNA of pelB-dspGLuc was cloned into NdeI and PstI sites of pRSET$_B$ (Invitrogen), yielding pRSET-pelB-dspGLuc. For mutant GLuc, The sequences corresponding to dspGLuc were randomly mutated by error-prone PCR, The sequence for dspGLuc in pRSET-pelB-dspGLuc was replaced with those PCR products containing mutant dspGLuc. These plasmids were transformed into JM109(DE3) competent cells, then, spread on LB ampicillin plates. After each bacterial colony was visible, each colony was picked and transferred to two new LB ampicillin plates, sequentially. After colonies were grown, we poured 50 μM coelenterazine containing PBS solution on top of the plate. The colonies which showed bright luminescence were picked and and evaluated by sequencing analysis. We obtained two mutants that showed bright luminescence. Mut1 contained K50E, M60L, M127I mutations, and Mut2 contained A36V, V113D mutations. We analyzed each of these mutations and found these mutations showed additive effects. A combination of these mutations resulted in an even brighter GLuc mutant, which had K50E, M60L, V113D, M127I and G184D mutations (GLucM23).



	A36	K50	M60	V113	M127	G184
wild	A	K	M	V	M	G
mut 1		E	L		I	
mut 2	V			D		
mut a		E				
mut b			L		I	
mut c(ref.3)			L		L	
mut d		E	L		L	
mut e	V					
mut f				D		
mut g						D
mut h				D		D
mut i	V			D		D
mut j	V	E	L		I	
mut k		E	L	D	I	
mut l	V	E	L	D	I	
mut m(mut23)		E	L	D	I	D
mut n	V	E	L	D	I	D

Figure S1. Displaying types of mutant variations of GLuc generated and their respective activities.

Mammalian cell expression constructs

To make pCDNA3- GLuc-Venus-KDEL, the entire sequence of GLuc was amplified by PCR with primers containing BamHI site, kozak sequence (ccacc) before start codon at the 5′ end and BspEI site, KDEL for endoplasmic reticulum localization signal sequence, stop codon and EcoRI site at the 3′ end. This PCR product was cloned into BamHI and EcoRI site of pcDNA3 vector (Invitrogen). This plasmid was then cut by BspEI and the cDNA of Venus fluorescent protein containing 5′ BspEI site and 3′ AgeI site was inserted. To make pDisplay-mKO-GLucM23, the entire sequence of mKusabira-Orange (mKO) was amplified by PCR with primer containing BamHI site at the 5′ end and BglII site at the 3′ end. The sequence of dspGLucM23 was also amplified by PCR with primers containing BglII site at the 5′ end and PstI site at the 3′ end. Two PCR products were ligated and cloned into BglII and PstI sites of pDisplay vector (Invitrogen). pDisplay-mKO-GLuc was constructed in a similar fashion.

General experimental details for cell-cultures

HeLaS3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen), supplemented with 10% fetal bovine serum (FBS) at 37°C under 5% CO_2. *Transfection*: Optimem (Invitrogen) solutions containing lipofectamine 2000

(Invitrogen) and plasmid DNA were added to HeLaS3 cell cultures and incubated at 37°C under 5% CO_2 for 24 h. Cells were then washed three times with PBS, trypsinized, washed with Leibovitz's L-15 medium, then resuspended in black 96-well optiplates (PerkinElmer) and incubated at 37 °C in luminometer (PerkinElmer).

Preparation of secreted *Gaussia* luciferase

HEK293T cells maintained in DMEM (no phenol red) supplemented with 10% FBS and transfected with pCM-GLuc (New England Biolabs) were incubated at 37 °C under 5% CO_2 for 24 h. The cell-medium was carefully transferred to a separate tube, and centrifuged. The supernatant was aliquoted and stored at −80 °C.

Preparation of *Renilla* luciferase cell lysate

HEK293T cells transfected with pRL-TK at 37 °C under 5% CO_2 for 24 h were washed with PBS (0.1 M, pH 7.4). 800 µL lysate buffer (20 mM Tris buffer (pH 7.4) 150 mM NaCl), containing a mixture of protease inhibitors (PSMF (1 mM), Leupeptin (~ 1 µg / ml), and Pepstatin (~ 1 µg / ml). The suspension was frozen in liquid nitrogen and dethawed 3 times, followed by centrifugation at 15000 rpm at 4 °C for 30 min. Protein concentration was determined by Bradford assay; 41.67 µg / mL.

Determination of Relative and Maximum Luminescence of Coelenterazine Derivatives with GLuc

To a solution of 10 µL GLuc in 90 µL PBS (pH 7.4, 20 mM EDTA, 0.02% Tween20) was added a buffer solution of coelenterate substrate (100 µL, 10 µM) via auto-injector (Final concentration 5 µM). Luminescence was measured for 60 s at 1 s intervals.

Determination of Relative and Maximum Luminescence of Coelenterazine Derivatives with RLuc

To a solution of Rluc (12.5 µg / mL) in 100 µL Tris buffer (20 mM , pH 7.6), 10 mM EDTA) was added a buffer solution of coelenterate substrate (100 µL, 10 µM) via auto-injector (Final concentration 5 µM). Luminescence was measured for 20 s at 1 s intervals.

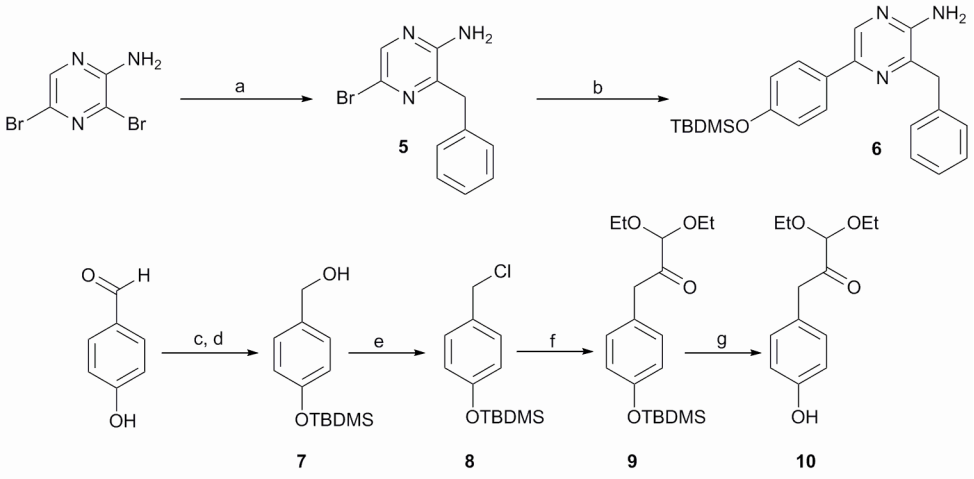
Bioluminescence Imaging

HeLaS3 cells in glass-bottom dishes were transfected with pDisplay-mKO-GLuc, pDisplay-mKO-GlucM23, or pcDNA3-GLuc-Venus-KDEL and incubated at 37°C under 5% CO_2 for 20 h. Cells were washed with Hank's balanced salt solution (HBSS) and suspended in 100 µL HBSS (covering the central glass bottom part of the dish). Coelenterazine or CoelPhos in HBSS (1000 µL, 25 µM) was added to the glass-bottom dish (final concentration 22.7 µM) and luminescence was

recorded at set exposure times. Fluorescence microscopic images were obtained before addition of coelenterate substrate (mKO: ex/em: 548/561; 200 ms; Venus Ex/Em: 515/528; 300 ms).

2. Syntheses of Compounds

1. Synthesis of coelenteramine and α-ketoacetal.



Scheme S1. (a) $PhCH_2MgCl$, $ZnCl_2$, $Pd(PPh_3)_2Cl_2$, THF, rt, 72 h, 83%; (b) 4-(TBDMS-O)Ph-$B(OH)_2$, bddp, $(C_6H_5CN)_2PdCl_2$, Na_2CO_3 (1M (aq)), EtOH, Toluene, reflux, 24 h, 83%; (c) TBDMSCl, imidazole, DMF, rt, 12 h; (d) $NaBH_4$, MeOH, 0 °C, 2 h, 86% over 2 steps; (e) $SOCl_2$, CH_2Cl_2, 0°, 2 h, 81%; (f) Mg, $EtBr_2$, THF, sonication; ethyl diethoxyacetate, THF, -78°C, 6 h, 47%; (g) Bu_4NF, THF, 0 °C, 30 min, 93%.

3-benzyl-5-bromo-2-pyrazinamine (5)

To a solution of benzylmagnesium chloride (2.0 M, 2.6 mL, 5.20 mmol) in THF (10 mL) was added zinc chloride in Et_2O (1.0 M, 5.7 mL, 5.70 mmol) at room temperature under argon. The resulting turbid mixture was stirred for 30 min, after which bis(triphenyl phosphine)palladium (II) dichloride (83 mg, 0.12 mmol) and 2-amino-3,5-dibromo-pyrazine (600 mg, 2.37 mmol) was added. The reaction was stirred for 3 days at room temperature. The reaction mixture was poured into water and extracted with EtOAc. The combined organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. Purification by silica gel chromatography (25% EtOAc / hexane) afforded 525 mg (1.99 mmol, 84%) of 3-benzyl-5-bromo -2-pyrazinamine (**5**). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.96 (s, 1H) 7.26-7.14 (m, 5H) 4.01 (s, 2H) ESI-MS: calc: 264.02; found: 264.00 $[M+H]^+$.

3-benzyl-5-(4-*tert*-butyldimethylsilyloxyphenyl)-2-pyrazinamine (6)

To a suspension of bis(benzonitrile)dichloro palladium (44 mg, 0.11 mmol) in toluene (5 mL) was added 1,4-bis(diphenyl-phosphino)butane (53 mg, 0.12 mmol) and the mixture was stirred for 30 min under argon at room temperature. 5-benzyl-5-bromo-2-pyrazinamine (**5**)(519 mg, 1.97 mmol), 4-(*tert*-butyldimethylsilyloxy)-phenylboronic acid (656 mg, 2.60 mmol), toluene (10 mL), ethanol (1 mL), and aqueous sodium carbonate (1 M) were added sequentially and the mixture was refluxed for 24 h. After cooling down to room temperature the reaction mixture was poured into water and extracted with EtOAc. The combined organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. Purification by silica gel chromatography (20-30% EtOAc / hexane) afforded 639 mg (1.63 mmol, 83%) 3-benzyl-5-(4-*tert*-butyldimethyl-silyloxyphenyl)-2-pyrazinamine (**6**) as a pale yellow solid. ^{1}H-NMR (400 MHz, $(CD_3)_2CO$) δ 8.36 (s, 1H) 7.88 (d, ^{3}J = 8.0 Hz, 2H) 7.35 (d, ^{3}J = 8.0 Hz, 1H) 7.28 (m, 2H) 7.20 (dd, ^{3}J = 8.0 Hz, 2H) 6.93 (d, ^{3}J = 8.0 Hz, 2H) 4.16 (s, 2H) 1.00 (s, 9H) 0.23 (s, 6H); ESI-MS: calc: 392.22; found: 392.18 $[M+H]^{+}$.

4-(*tert*-butyldimethylsilyloxy)phenylmethanol (7)

To a solution of 4-hydroxybenzaldehyde (5.75 g, 47.1 mmol) and imidazole (6.45 g, 94.8 mmol) in anhydrous CH_2Cl_2 (60 mL) was added TBDMSCl (7.88 g, 52.28 mmol) and was stirred overnight at room temperature under an argon atmosphere. The reaction mixture was poured into water washed with water and brine. The organic layer was dried over anhydrous sodium sulfate and the solvent was removed *in vacuo* and the residue was dried over night. To a solution of the crude 4-(*tert*-butyldimethylsilyloxy)benzaldehyde in methanol was added sodium borohydride (2.24 g, 59.1 mmol) at 0 °C. The ice-bath was removed and the solution was stirred under argon for 2 h. Reaction was quenched by addition of brine. MeOH was evaporated and water was added. The aqueous layer was extracted with EtOAc. The organic layer was washed with brine, dried over sodium sulfate and concentrated *in vacuo,* followed by purification by silica gel chromatography (30% EtOAc / hexane) to afford 9.709 g (86% for 2 steps) of 4-(*tert*-butyldimethylsilyloxy)phenylmethanol (**7**) as a pale yellow viscous oil. ^{1}H-NMR (400 MHz,$CDCl_3$) δ 7.22 (d, ^{3}J = 8.6 Hz, 2H) 6.82 (d, ^{3}J = 8.6 Hz, 2H) 4.60 (s, 2H) 0.99 (s, 9H) 0.20 (s, 6H).

4-(*tert*-butyldimethylsilyloxy)benzyl chloride (8)

To solution of 4-(*tert*-butyldimethylsilyloxy)phenylmethanol (**7**)(10.6 g, 41.3 mmol) in CH_2Cl_2 (~50 mL) at 0 °C under argon was added thionyl chloride (4.6 mL, 63.0 mmol) dropwise. The reaction mixture was stirred for 2 h and poured into water. The organic layer was washed with water ample times, followed by brine. The organic layer was dried over sodium sulfate and evaporated *in vacuo*. Purification by silica gel chromatography (10% EtOAc / hexane) to afford 8.60 g (81 %) of 4-(*tert*-butyl-dimethylsilyloxy)benzyl chloride (**8**). ^{1}H-NMR (400 MHz,$CDCl_3$) δ 7.26 (d, ^{3}J = 8.6 Hz, 2H) 6.83 (d, ^{3}J = 8.6 Hz, 2H) 4.57 (s, 2H) 1.00 (s, 9H) 0.22 (s, 6H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 155.89, 130.29, 129.96, 120.26, 46.29, 25.64, 18.19, -4.44.

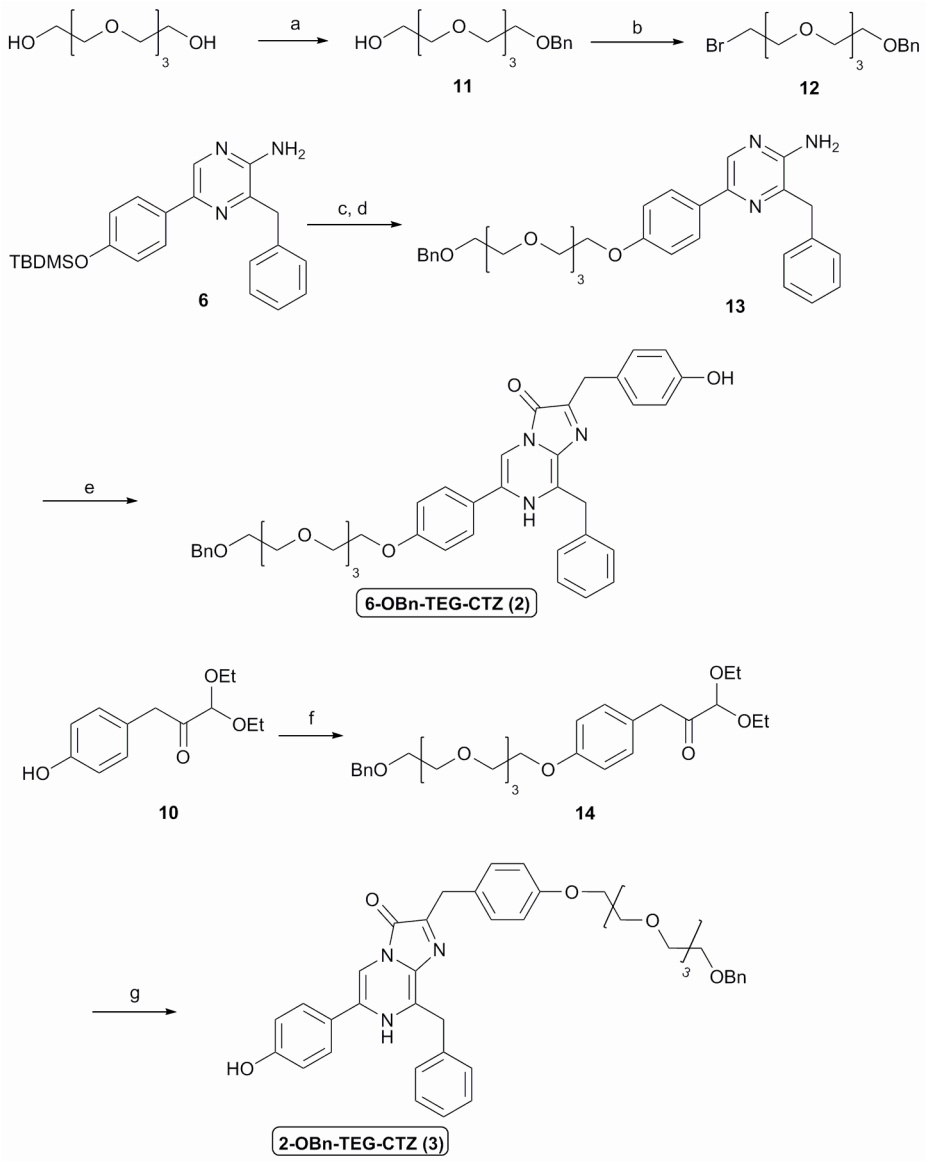
3-(4-(*tert*-butyldimethylsilyloxy)phenyl)-1,1-diethoxyacetone (9)

To magnesium turnings (4.29 g, 176.5 mmol) (vigorously stirred under argon for 3 days) was added 4-(*tert*-butyldimethyl-silyloxy)benzyl chloride (**8**)(8.60 g, 33.5 mmol) in anhydrous THF, followed by additional THF (~ 50 mL), and 1,2-dibromo-ethane (50 µL, 0.58 mmol). The reaction mixture was sonicated for 5 mins, and heated at 50 °C for 1 h. The dark grey reaction mixture was allowed to cool to room temperature. To a separate reaction flask was added ethyl diethoxyacetate (8.0 mL, 44.7 mmol) and THF (~10 mL) and cooled to -78 °C (dry ice / acetone) under argon. The Grignard reagent was added via syringe over 30 mins and the reaction mixture was allowed to stir for 6 h, followed by addition of water (25 mL) and the reaction mixture allowed to warm to room temperature. Additional water was added and extracted with EtOAc. The organic layer was washed with brine, dried over sodium sulfate, and concentrated *in vacuo*. The crude pale yellow oil was purified by silica gel chromatography (10% EtOAc / hexane) to afford 5.54 g (15.7 mmol, 47%) of 3-(4-(*tert*-butyldimethylsilyloxy)-phenyl)-1,1-diethoxy acetone (**9**) as a pale yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.07 (d, ^{3}J = 8.0 Hz, 2H) 6.78(d, ^{3}J = 8.0 Hz, 2H) 4.62 (s, 1H) 3.80 (s, 2H) 3.70-3.51 (m, 2 x 2H) 1.24 (t, ^{3}J = 7.0 Hz, 6H) 0.97 (s, 9H) 0.18 (s, 6H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 203.6, 154.5, 130.7, 126.3, 120.1, 102.1, 63.3, 43.1, 25.7, 18.2, 15.1, -4.5. ESI-MS: calc: 370.2408; found: 370.3069 $[M+NH_4]^+$.

3-(4-hydroxyphenyl)-1,1-diethoxyacetone (10)

To 0 °C cooled solution of 3-(4-(*tert*-butyldimethylsilyloxy)phenyl)-1,1- diethoxy acetone (**9**)(1.50 g, 4.25 mmol) in THF (10 mL) under argon was added tetra-*n*-butylammonium fluoride (1.0 M in THF, 2.2 mL, 2.2 mmol). After 30 min, the reaction was quenched with saturated aqueous sodium chloride and extracted with CH_2Cl_2. The combined organic layer was dried over anhydrous sodium sulfate and concentrated *in vacuo*. Purification by silica gel chromatography afforded 944 mg, (3.96 mmol, 93%) of 3-(4-hydroxyphenyl)-1,1-diethoxyacetone. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.26 (d, ^{3}J = 8.0 Hz, 2H) 6.78 (d, ^{3}J = 8.0 Hz, 2H) 4.69 (s, 1H) 4.63 (s, 2H) 3.82 (s, 2H) 3.72-3.53 (m, 2 x 2H) 1.25 (t, 6H). ESI-MS: calc: 256.1543; found: 256.1663 $[M+NH_4]^+$.

2. Synthesis of 2 and 6-OBn-TEG-CTZ.



Scheme S2 Synthesis of 6-OBn-TEG-CTZ and 2-OBn-TEG-CTZ. (a) NaH, BnBr, THF/DMF, 0 °C to rt. 5 h, 67%; (b) PPh_3, CBr_4, CH_2Cl_2, reflux, 12 h, 91%; (c) Bu_4NF, THF, 0 °C, 2 h; (d) **12,** NaH, DMF, 0 °C, 5 h, 55% over 2 steps, (e) **9**, 1,4-dioxane / 6N HCl (10:1), 24 h, 49%; f) **12**, Cs_2CO_3, MeCN, reflux, 3 h, 73%; (g) **6**, 1,4-dioxane / 6N HCl (10:1), reflux, 14 h, 17%.

13-bromo-1-phenyl-2,5,8,11-tetraoxatridecane (12)

Sodium hydride (60% in mineral oil)(839 mg, 20.98 mmol) was added to a cooled reaction flask containing anhydrous DMF under argon. The mixture was stirred for 10 minutes, after which the ice-bath was removed and the mixture was stirred at rt for another 15 min. The mixture was cooled with an icebath, followed by dropwise addition of benzyl bromide (2.3 mL, 19.34 mmol). Reaction mixture was concentrated *in vacuo*, followed by silica gel chromatographyEtOAc to afford **11** (3.34 g, 11.61 mmol, 67%) as a colorless oil. To a solution of triphenylphosphine (856 mg, 3.26 mmol) and carbon tetrabromide (1088 mg,

3.28 mmol) in anhydrous CH_2Cl_2 under argon was added **7** (592 mg, 2.08 mmol). The reaction mixture was refluxed for 18 h. Reaction mixture was concentrated *in vacuo*. silica gel chromatography ((1:1) EtOAc / hexane) to afford **12** (483 mg, 15.7 mmol, 79% over two steps) as a colorless oil. 1H-NMR (400 MHz, $CDCl_3$) δ 7.35-7.34 (m, 5H) 4.57 (s, 2H) 3.72-3.59 (m, 16H). FAB: Calculated: 540.0879; Found: 540.0887 $[M+Na]^+$. ESI-MS: Calculated: 540.0879; Found: 540.1243$[M+Na]^+$.

3-benzyl-5-(4-(1-phenyl-2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)pyrazin-2-amine (13)

To an ice-cooled solution of 3-benzyl-5-(4-*tert*-butyldimethylsilyloxyphenyl)-2-pyrazinamine (**6**) (80 mg, 0.20 mmol) in anhydrous THF under argon was added tetrabutylammonium fluoride (160 µL, 0.16 mmol) in THF. After 1 stirring for 1 h the reaction was quenced with brine, poured into water and extracted with EtOAc. The combined organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The resulting residue was subjected to silica gel chromatography ((1:1) EtOAc / Hexane), affording crude coelenteramine (~56 mg, 0.2 mmol). To a ice-cooled solution of sodium hydride (60% in mineral oil)(18 mg, 0.45 mmol) in anhydrous DMF (5 mL) was added coelenteramine (55 mg, 0.2 mmol) and the reaction mixture was stirred for 30 min. 12-bromo-1-benzyl-tetraethylene glycol (170 mg, 0.49 mmol) in DMF (5 mL) was added dropwise. The ice-bath was removed and the reaction mixture was stirred at rt for 4.5 h, after which the reaction was quenched with 20% NH_4Cl. The reaction mixture was diluted with water and extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The resulting residue was subjected to silica column chromatography (2% MeOH / CH_2Cl_2) to afford the desired product **13** (58 mg, 55% over two steps). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.31(s, 1H) 7.85 (d, 2H, ^3J = 8.0 Hz) 7.33-7.25 (m, 10H) 6.98 (d, ^3J = 8.0 Hz, 2H) 4.55 (s, 2H) 4.46 (s, 2H) 3.86 (t, 2H) 3.74-3.61 (m, 14H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 158.90, 151.29, 142.38, 140.40, 138.22, 136.82, 136.75, 130.10, 128.91 128.55, 128.32, 127.71, 127.56, 126.96, 126.90, 114.87, 73.18, 70.81, 70.61, 69.68, 69.39, 67.44, 41.15. ESI-MS: Calculated: 544.2806; Found: 544.2834 $[M+H]^+$. FAB: Calculated: 543.2733; Found: 543.2722 $[M]^+$.

1,1-diethoxy-3-(4-(1-phenyl-2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)propan-2-one (14)

A solution of 3-(4-hydroxyphenyl)1,1-diethoxyacetone (**10**) (100 mg, 0.42 mmol), Cs_2CO_3 (202 mg, 0.62 mmol), and **12** (217 mg, 0.62 mmol) in anhydrous MeCN (5 mL) was refluxed under argon for 3 h. The reaction mixture was poured into water and extracted with EtOAc. The combined organic layer was washed with brine and dried over anhydrous sodium sulfate. The mixture was concentrated *in vacuo*, followed by purification by silica gel chromatography (40% EtOAc / Hexane) to yield **14** (154 mg, 0.31 mmol, 73%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.34-7.33 (m, 5H) 7.11 (d, ^3J = 8.4 Hz, 2H) 6.85 (d, ^3J = 8.4 Hz, 2H) 4.62 (s, 1H) 4.56 (s, 2H) 4.10 (t, 2H) 3.83 (t, 2H) 3.81 (s, 2H) 3.73-3.67 (m, 12H) 3.68-3.52 (m, 4H) 1.24 (t, ^3J = 7.2 Hz, 6H). ^{13}C-NMR (100 MHz,$CDCl_3$) δ 203.47, 157.69, 138.21, 130.65, 128.30, 127.68, 127.52, 125.83, 114.60, 102.14, 73.16, 70.75, 70.59, 69.66, 69.37, 67.13, 63.26, 42.80, 15.11. ESI-MS: Calculated: 522.3061; Found: 522.2361 $[M+NH_4]^+$.

6-OBn-TEG-CTZ (2)

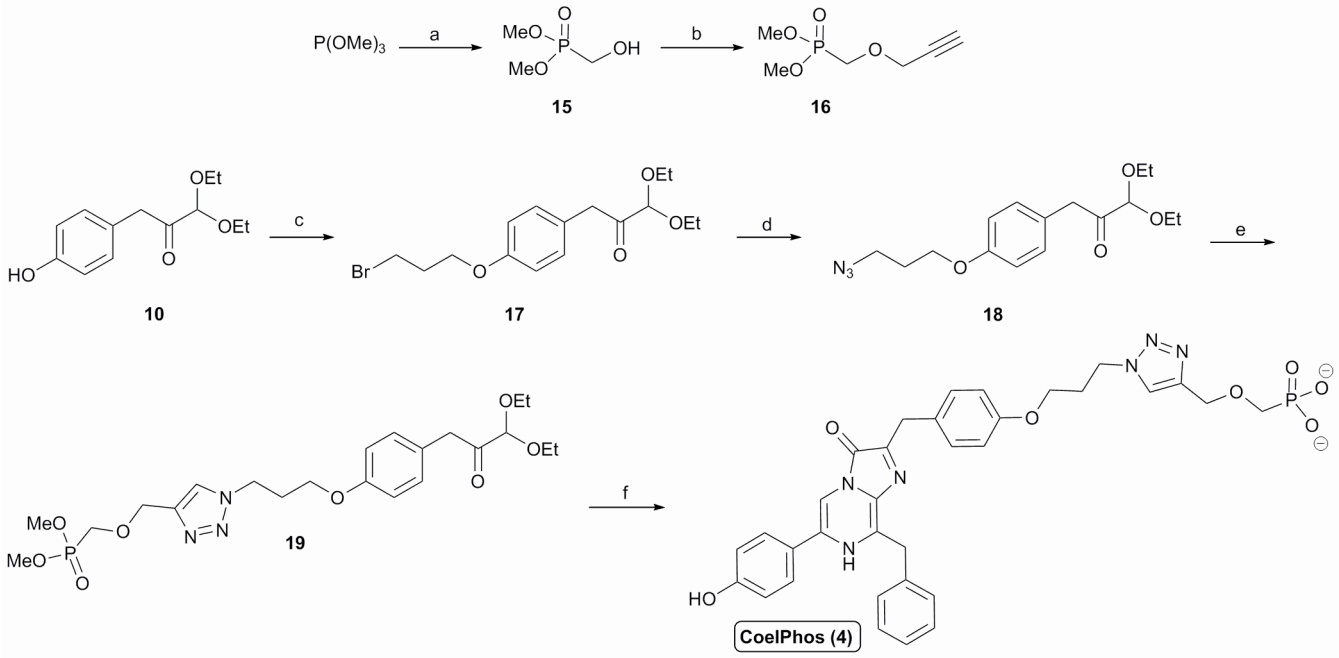
A solution of **13** (55 mg, 0.10 mmol) and **9** (73 mg, 0.21 mmol) in 1,4-dioxane (5 mL) and 6N HCl (0.5 mL) was refluxed under argon for 24 h. The reaction was allowed to cool to rt and concentrated *in vacuo*. Silica gel chromatography, (3-6 % MeOH / CH_2Cl_2) afforded the desired product **6-OBn-TEG-CTZ (2)** as a dark red solid (14 mg, 0.02 mmol, 20 %). ^{1}H-NMR (400 MHz, CD_3OD) δ 7.67 (s, 1H) 7.38 (m, 2H) 7.31-7.15 (m, 10H) 6.78 (d, ^3J = 8.4 Hz, 2H) 6.72 (d, ^3J = 8.0 Hz, 2H) 4.52 (s, 2H) 4.41 (s, 2H) 4.13 (m, 2H+2H) 3.85 (t, 2H) 3.70-3.61 (m, 12H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 160.77, 156.27, 138.76, 136.92, 131.35, 130.90, 130.45, 129.85, 129.48, 129.33, 128.93, 128.81, 128.44, 128.28, 127.81, 125.91, 115.87, 115.64, 73.78, 73.12, 71.29, 71.11, 70.25, 70.05, 68.18, 61.78, 30.25. ESI-MS: Calculated: 690.3174; Found: 690.3279 $[M+H]^+$.

2-OBn-TEG-CTZ (3)

A solution of **2** (51 mg, 0.13 mmol) and **10** (124 mg, 0.25 mmol) in 1,4-dioxane (5 mL) and 6N HCl (0.5 mL) was refluxed under argon for 14 h. The reaction was allowed to cool to rt and concentrated *in vacuo*. Silica gel chromatography, (5-10 % MeOH / CH_2Cl_2) afforded the crude desired product (38.5 mg). The residue was further purified via RP-HPLC (H_2O (0.1 % formic acid) / MeCN (0.1 % formic acid) ; 60:40 → 70:30, 4.7 mL/min; column: ODS-3). The combined fractions were lyophilized, yielding the desired product **2-OBn-TEG-CTZ (3)** as a red solid (15 mg, 0.02 mmol, 17%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.64 (s, 1H) 7.32 (m, 2H) 7.25-7.07 (m, 10H) 6.98 (d, 2H, ^3J = 8.4 Hz) 6.72 (d, ^3J = 8.0 Hz, 2H) 4.54 (s, 2H) 4.41 (s, 2H) 4.02 (m, 2H + 2H) 3.84 (t, 2H) 3.68-3.58 (m, 12H) ^{13}C-NMR (100 MHz, $CDCl_3$) δ 158.77, 155.35, 137.56, 134.92, 129.64, 130.40, 130.25, 129.85, 129.68, 129.26 128.73, 128.51, 128.34, 128.26, 127.14, 125.68, 115.41, 114.55, 73.67, 73.08, 71.24, 71.01, 70.04, 70.01, 68.28, 60.75, 30.15. ESI-MS: Calculated:690.3174; Found: 690.2411 $[M+H]^+$. FAB: Calculated: 689.3101; Found: 689.3088 $[M]^+$.

3. Synthesis of CoelPhos

Comment: Initially installing of a propylphosphonate at the 2-position of coelenterazine was perceived as a suitable candidate compound. However, alkylation of the α-ketoacetal **10** with 3-bromopropylphosphonate proved difficult with formation of several side products (data not shown). Interestingly alkylation of **10** with 1,3-dibromopropane gave **17** which could be separated from the undesired side products more easily. Alkylation of **10** with ethyl 4-bromobutyrate has been reported previously,[1] which also resulted in a poor yield. On the other hand, alkylation of **10** with PEG linker **12** gave **14** in a satisfactory yield, suggesting stereochemistry to be an important factor in the general alkylation of **10**.. Hence we modified our synthetic route by considering converting the propyl bromide **17** into the propyl azide **18**, which would allow for diversification via copper(I)-catalyzed azide-alkyne Huisgen cycloaddition. In our initial attempt to form the 1,2,3-triazole between **16** and **18**, we utilized the common solvent system H_2O/*t*BuOH (1:1),[4] however in our hands hardly any product was formed. When we applied a two-phase solvent system (H_2O/CH_2Cl_2 (1:1)), which has been shown to improve reaction kinetics,[5] gave the desired 1,2,3-triazole **19** in very good yield.



Scheme S3. Synthesis of CoelPhos. (a) paraformaldehyde, TEA, 130 °C, 3 h. 12%; (b) propargyl bromide, NaH, THF, -70 °C. 35%; (c) 1,3-dibromopropane, Cs_2CO_3, MeCN, reflux, 3 h. 35%; (d) NaN_3, DMF, 50 °C, 12 h, 76%; (e) **16**, $CuSO_4$, Sodium Ascorbate, H_2O/CH_2Cl_2(1:1), rt, 12 h, 88%; (f) **6**, 1,4-dioxane/6N HCl (10:1), 110 °C, 5-6 h, 9%.

Dimethyl hydroxymethylphosphonate (15)

To a solution of paraformaldehyde (1.27 g, 42.4 mmol) in trimethyl phosphite (5 mL, 42.3 mmol) was added triethylamine (0.6 mL, 4.30 mmol) under argon and was refluxed at 130 °C for 3 h. Volatiles were removed *in vacuo*. The crude residue was purified by silica gel chromatography (4-5% MeOH / EtOAc) to afford 697 mg (4.98 mmol, 12%) of dimethyl hydroxyl-methylphophonate (**15**). ^{1}H-NMR (400 MHz,$CDCl_3$) δ 3.96 (m, 2H) 3.81 (d, ^{3}J = 8 Hz, 6H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 56.4 (d, J = 162.1 Hz) 53.1 (d, J = 6.6 Hz); ^{31}P-NMR (162 MHz, $CDCl_3$) single peak. ESI-MS: calc: 141.0311, found: 141.0473 $[M+H]^+$.

Dimethyl (prop-2-ynyloxy)methylphosphonate (16)

To -78 °C cooled solution of dimethyl hydroxymethylphosphonate (**15**)(172 mg, 1.23 mmol) in THF (2 mL) was added sodium hydride (96 mg, 2.40 mmol) and allowed to stir for 1.5 h. A solution of propargyl bromide (0.2 mL, 1.85 mmol) in THF (1 mL) was added dropwise via syringe. The reaction was left stirring for 14 h. The reaction was quenched with ammonium chloride and the solvent was evaporated. Water was added and extracted with CH_2Cl_2. The combined organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. Purification by silica gel chromatography (3-4% MeOH / EtOAc) afforded 76 mg (0.43 mmol, 35%) of dimethyl (prop-2-ynyloxy)methylphosphonate (**16**) as a colorless oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.28 (m, 2H) 3.92 (d, ^{2}J = 12Hz, 2H) 3.82 (d, ^{3}J = 8 Hz, 6H) 2.51 (m, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 78.1, 76.0, 63.2 (d, J = 167.1 Hz) 60.1 (d, J = 14.9 Hz) 53.1 (d, J = 6.5 Hz); ^{31}P-NMR (162 MHz, $CDCl_3$) single peak. ESI-MS: calc: 179.0468, found: 179.0625 $[M+H]^+$.

3-(4-(3-bromopropoxy)phenyl)-1,1-diethoxyacetone (17)

To a solution of 3-(4-hydroxyphenyl)-1,1-diethoxyacetone (**10**) (952 mg, 4.00 mmol) and Cs_2CO_3 (1411 mg, 4.33 mmol) in anhydrous acetonitrile (20 mL) under argon was added 1,3-dibromopropane (2 mL, 19.7 mmol) by syringe. The solution was heated at 100 °C for 3 h. After cooling to room temperature the reaction mixture was filtered and washed with EtOAc and evaporated to dryness. The resulting residue was partitioned between water and EtOAc and the aqueous layer was extracted with EtOAc. The combined organic layer was washed with brine, dried over anhydrous sodium sulfate and concentrate *in vacuo*. Purification by gradual silica gel chromatography (5-10% EtOAc / hexane) afforded 510 mg (1.42 mmol, 35%) of 3-(4-(3- bromopropoxy)phenyl)-1,1-diethoxyacetone (**17**) as a pale yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.12 (d, ^{3}J = 8.0 Hz, 2H) 6.86 (d, ^{3}J = 8.0 Hz, 2H) 4.63 (s, 1H) 4.09 (t, ^{3}J = 8.0 Hz, 2H) 3.83 (s, 2H) 3.70 (m, 2H) 3.60 (m, 2H) 3.55 (m, 2H) 2.31 (m, 2H) 1.25 (t, ^{3}J = 8.0 Hz, 6H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 203.5, 157.6, 130.8, 126.0, 114.6, 102.3, 65.2, 63.4, 42.8, 32.4, 30.1, 15.2.

3-(4-(3-azidopropoxy)phenyl)-1,1-diethoxyacetone (18)

To a solution of 3-(4-(3- bromopropoxy)phenyl)-1,1-diethoxyacetone (**17**) (268 mg, 0.75 mmol) in anhydrous DMF, (~2 mL) was added sodium azide (78 mg, 1.20 mmol) and the reaction was heated at 50 °C for 12 h. The reaction was allowed to cool to room temperature, poured into water, and extracted with EtOAc. The combined organic layer was washed with brine and dried over anhydrous sodium sulfate, and concentrated *in vacuo*. Purification by silica gel chromatography (10% EtOAc / hexane) afforded 187 mg (0.58 mmol, 76%) of 3-(4-(3-azidopropoxy)phenyl)-1,1-diethoxyacetone (**18**). ^{1}H-NMR (400 MHz,$CDCl_3$) δ 7.12 (d, ^{3}J = 8.0 Hz, 2H) 6.85 (d, ^{3}J = 8.0 Hz, 2H) 4.62 (s, 1H) 4.03 (s, 2H) 3.70 (t, ^{3}J = 7.2 Hz, 2H) 3.54 (m, 4H) 2.04 (t, ^{3}J = 5.6 Hz, 2H) 1.25 (t, ^{3}J = 6.6 Hz, 6H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 203.5, 157.6, 130.8, 126.0, 114.6, 102.3, 64.5, 63.4, 48.3, 42.8, 28.8, 15.2. ESI-MS: calc: 339.2027; found: 339.2164 $[M+NH_4]^+$.

Dimethyl((1-(3-(4-(3,3-diethoxy-2-oxopropyl)phenoxy)propyl)-1H-1,2,3-triazol- 4-yl)methoxy)methylphosphonate (19)

To a solution of 3-(4-(3-azidopropoxy)phenyl)-1,1-diethoxyacetone (**18**) (94 mg, 0.29 mmol), dimethyl (prop-2-ynyloxy) methylphosphonate (**16**) (61 mg, 0.34 mmol) in water (1 mL) and dichloromethane (1 mL) was added copper (II) sulfate (2.8 mg, 0.02 mmol), and sodium ascorbate (13.6 mg, 0.09 mmol). The reaction mixture was vigorously stirred for 12 h, poured into water and extracted with dichloromethane, dried over sodium sulfate, and concentrated *in vacuo*. Purification by silica gel chromatography afforded 129 mg (0.26 mmol, 88%) of dimethyl ((1-(3-(4-(3,3-diethoxy-2-oxopropyl)phenoxy)propyl)-1H-1,2,3-triazol- 4-yl)methoxy)methylphosphonate (**19**) as a viscous oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.60 (s, 1H) 7.12 (d, ^{3}J = 8.4 Hz, 2H) 6.83 (d, ^{3}J = 8.4 Hz, 2H) 4.75 (s, 2H) 4.63 (s, 1H) 4.58 (t, ^{3}J = 7.2 Hz, 2H) 3.96 (t, ^{3}J = 5.6 Hz, 2H) 3.88-3.83 (m, 4H) 3.78 (d, ^{3}J = 10.8 Hz, 6H) 3.71 (m, 2H) 3.56 (m, 2H) 2.39 (m, 2H) 1.25 (t, ^{3}J = 6.8 Hz, 6H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 203.4, 157.3, 143.8, 130.9, 126.3, 123.5, 102.33, 66.2, 63.2 (d, ^{3}J = 167.0 Hz) 63.9, 63.4, 53.0, 47.1, 42.6, 29.9, 15.1. ^{31}P-NMR (162 MHz, $CDCl_3$) single peak. ESI-MS: calc: 500.2156; found: 500.1754 $[M+H]^+$.

((1-(3-(4-((8-benzyl-6-(4-hydroxyphenyl)-3-oxo-3,7-dihydroimidazo[1,2-a]pyrazin-2-yl)methyl)phenoxy)propyl)-1H-1,2,3-triazol-4-yl)methoxy)methylphosphonate (CoelPhos)

To a solution of **19** (80 mg, 0.16 mmol) in 1,4-dioxane (1 mL) was added **6** (63 mg, 0.16 mmol). To this mixture was added 6 N $HCl_{(aq)}$ (100 µL) and the solution was thoroughly flushed and kept under an argon atmosphere. The reaction mixture was then heated to 110 °C and stirred for 5-6 h. The dark mixture was allowed to cool to room temperature and the solvent was evaporated. Reaction mixture was purified by reverse-phase HPLC (100 mM triethylammonium acetate / acetonitrile; 90:10 → 40:60, 4.7 mL/min; column: ODS-3). Collected fractions were concentrated, combined, and lyophilized to give product **CoelPhos** as the triethylammonium salt (11 mg, 9% yield). ^{1}H-NMR (400 MHz, CD_3OD) δ 7.94 (s, 1H) 7.70 (s, 1H) 7.50

(d, ^{3}J = 8.8 Hz, 2H) 7.35 (d, ^{3}J = 7.6 Hz, 2H) 7.23-7.11 (m, 7H) 6.82 (d, ^{3}J = 8.8 Hz, 2H) 6.75 (d, ^{3}J = 9.0 Hz, 2H) 4.62 (s, 2H) 4.53 (t, ^{3}J = 7.0 Hz, 2H) 4.34 (s, 2H) 4.04 (s, 2H) 3.89 (t, ^{3}J = 6.0 Hz, 2H) 3.56 (d, ^{3}J = 9.6 Hz, 2H) 2.28 (m, 2H). ^{13}C-NMR (100 MHz, CD_3OD) δ 159.52, 158.50, 146.07, 139.17, 133.63, 130.82, 129.85, 129.50, 129.14, 128.57, 127.73, 125.44, 125.40, 116.84, 115.45, 108.48, 69.43, 67.85, 66.68, 66.54, 65.57, 47.53, 36.71, 33.02, 31.09, 29.43, 23.85, 9.26. HRMS:ESI-MS: calc: 657.2221; found: 657.3062 $[M+H]^{+}$. ^{31}P NMR (162 MHz, CD_3OD, single peak. H-H COSY (400 MHz, CD_3OD)(Figure S2)

3. Supporting Figures

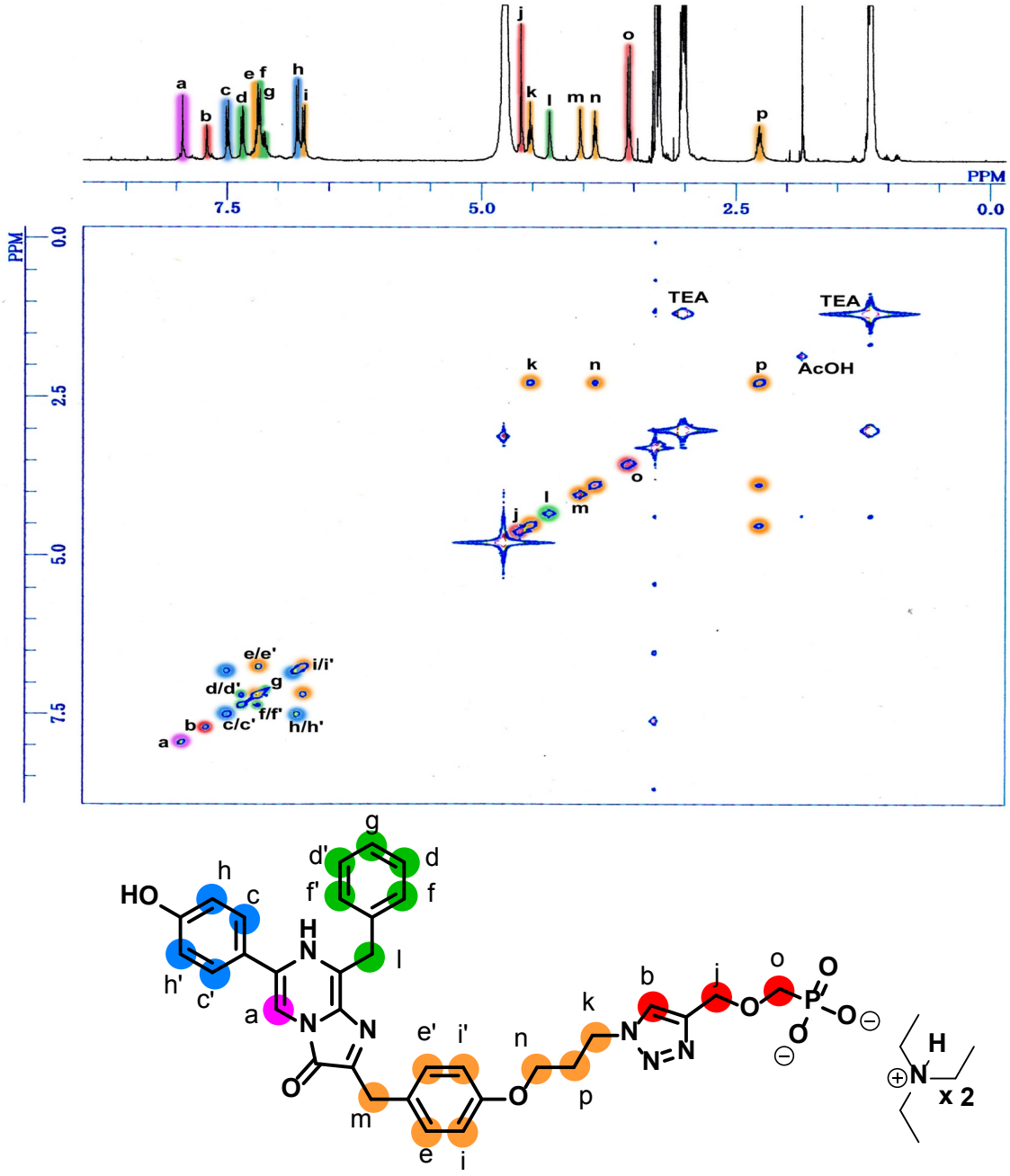


Figure S2. H-H-COSY diagram of CoelPhos

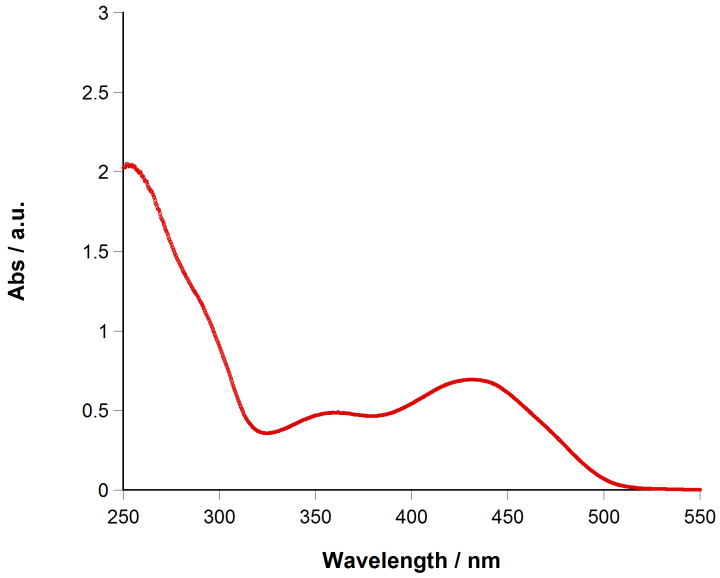


Figure S3. Absorbance spectrum of CoelPhos (90 µM in MeOH)

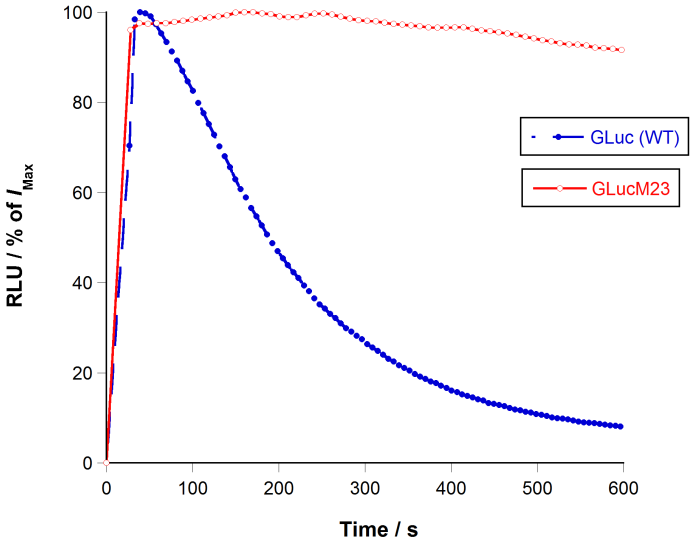


Figure S4. Luminescence profile of mutant GLucM23 in comparison with wild-type GLuc in living HEK293T cells. Reaction conditions: HEK293T cells were transfected with pDisp-mKO-GLucM23 or pDisp-mKO-GLuc. 1×10^4 cells were suspended in Leibovitz's L-15 medium (100 µL / well) in a multiwell plate. 100 µL solution of Coelenterazine in PBS (final concentration 25 µM) was added. Luminescence was measured for ~600 s at 10 s intervals at 37 °C in a luminometer.

Table S1. Relative and maximum luminescence of CoelPhos with *Renilla* luciferase cell lysate.

Compound	*Renilla* luciferase	
	I_{Total} (%)[a]	I_{Max} (%)[b]
Coelenterazine	100	100
CoelPhos	0.14	0.09

[a] I_{Total}: total luminescence integrating for 20 s in 1 s intervals.
[b] I_{Max}: maximum observed intensity at any 1 s interval.

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