Supporting Information for

Spirohexene-Tetrazine Ligation Enables Bioorthogonal Labeling of Class B G Protein-Coupled Receptors in Live Cells

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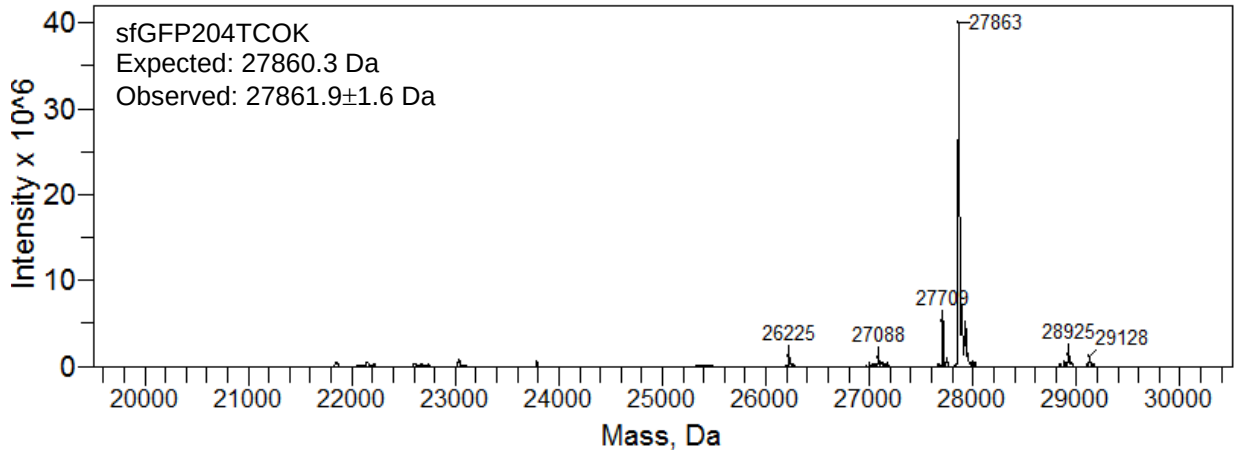
Supplemental Figures and Tables

a)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Mass, Da
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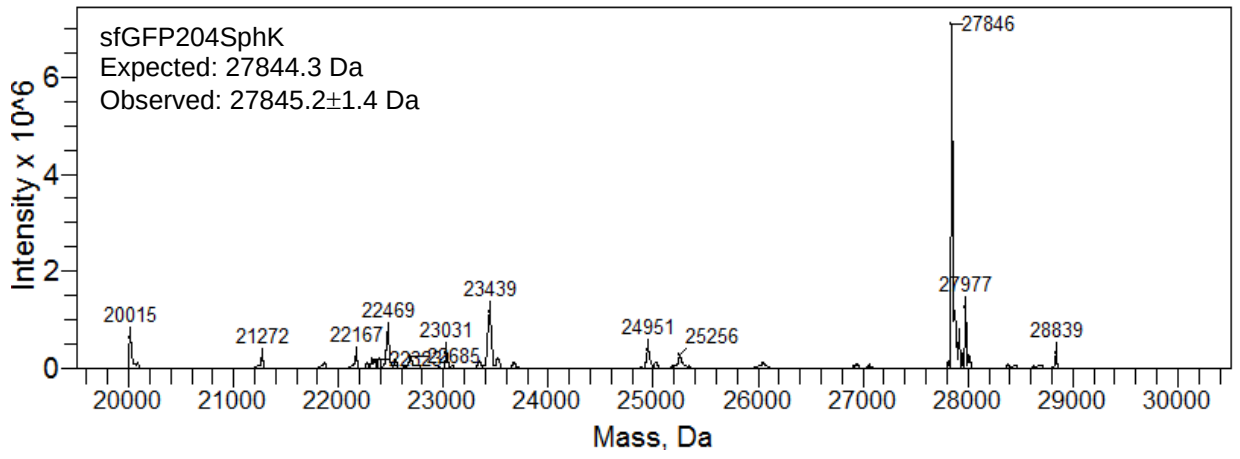
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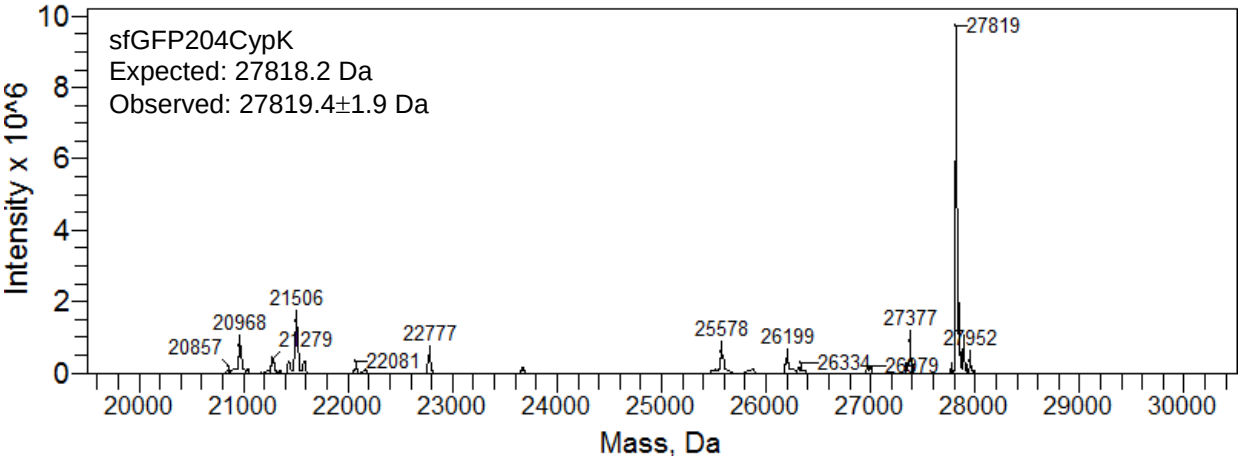
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b)

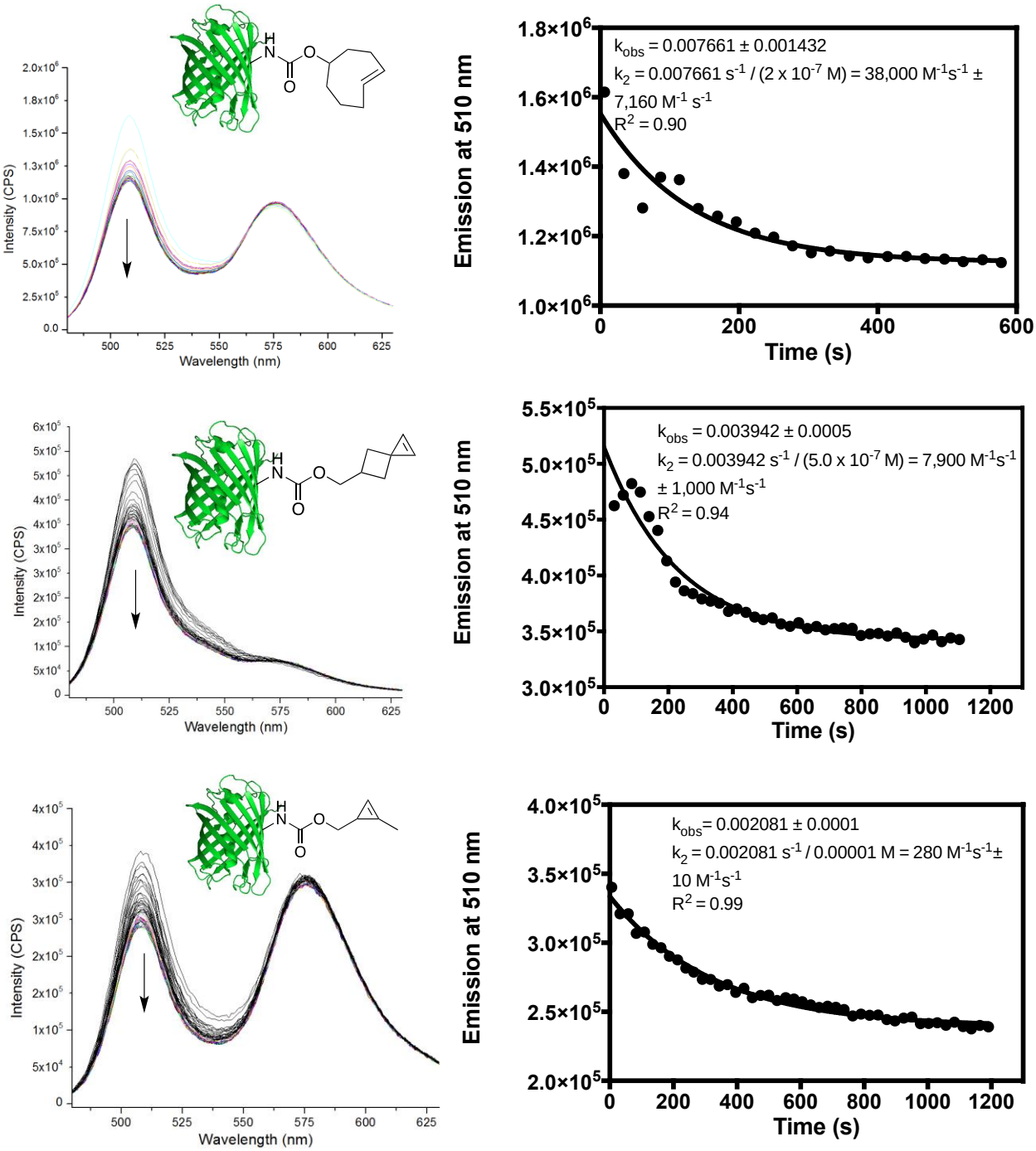


Figure S1. Fluorescence-based kinetic analysis of the ligation reaction between sfGFP encoding a strained alkene and DpTz-TAMRA (**6**). (a) Deconvoluted mass spectra of the recombinantly expressed and purified sfGFP204TCOK, sfGFP204SphK, and sfGFP204CypK. (b) Time courses of the fluorescence spectra of the reaction mixtures. Excitation was set at 460 nm and the emission window was set at 480-630 nm. For ligation with sfGFP204TCOK, 50 nM sfGFP204TCOK was mixed with 200 nM DpTz-TAMRA in phosphate buffer, *p*H 7.4. For

ligation with sfGFP204SphK, 100 nM sfGFP204SphK was mixed with 500 nM DpTz-TAMRA in phosphate buffer, *p*H 7.4. For sfGFP204CypK, 100 nM sfGFP204CypK was mixed with 10 μM DpTz-TAMRA in phosphate buffer, *p*H 7.4. The time-dependent decrease in GFP emission at 510 nm was plotted, and the data were fitted to an exponential rise equation in the GraphPad Prism 6.0 program: $y = (y_0\text{-a})\ e^{-kobs*t} + a$. The second-order rate constant of the ligation reaction, k_2, was calculated using the equation: $k_2 = k_{obs}/[\text{DpTz-TAMRA}]$.

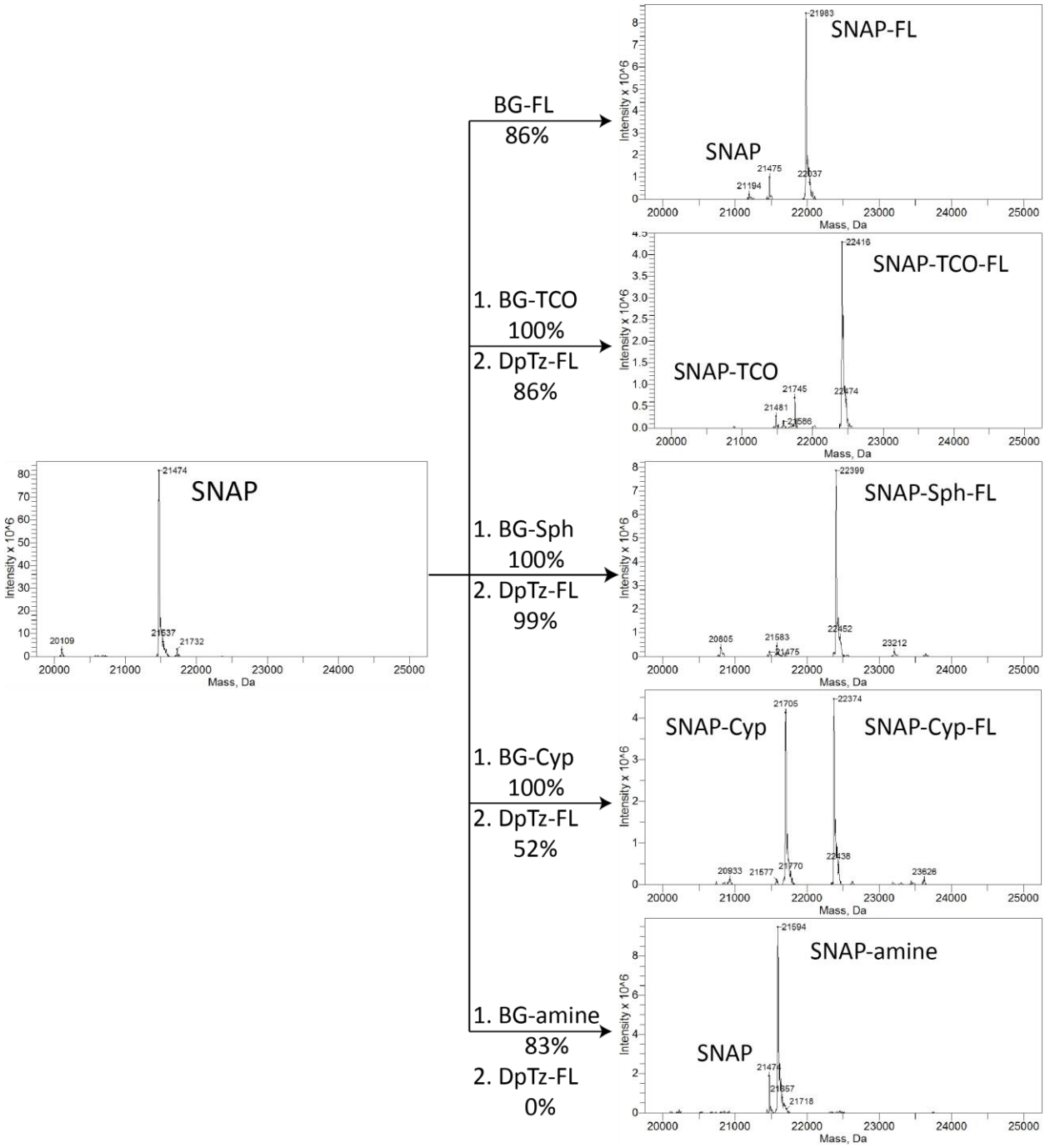
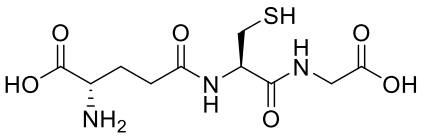
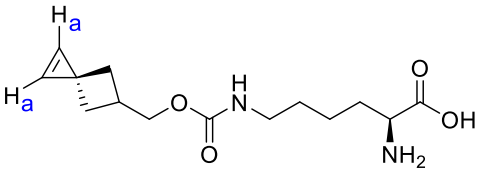


Figure S2. LC/ESI-MS analysis of the reaction of the strained alkene-bearing SNAP-tag protein with tetrazine. The SNAP-tag protein (5 μM) was labeled with BG-substrate (10 μM) at 37°C for 30 min and excess BG-substrate was removed through filtration. The labeled SNAP-tag was then reacted with fluorescein-tetrazine DpTz-FL (100 μM) at room temperature for 10 min. After filtration, the samples were analyzed by mass spectrometry. Reaction yield was calculated based on ion counts using the following equation: % conversion $= \frac{I_{SNAP-Tz}}{I_{SNAP-alkene} + I_{SNAP-Tz}} \times 100\%$.

a)



GSH (50 mM)



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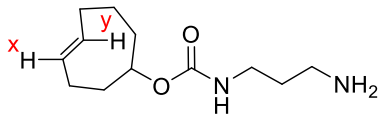
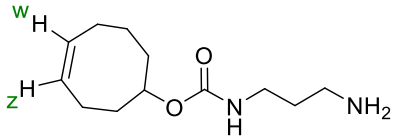
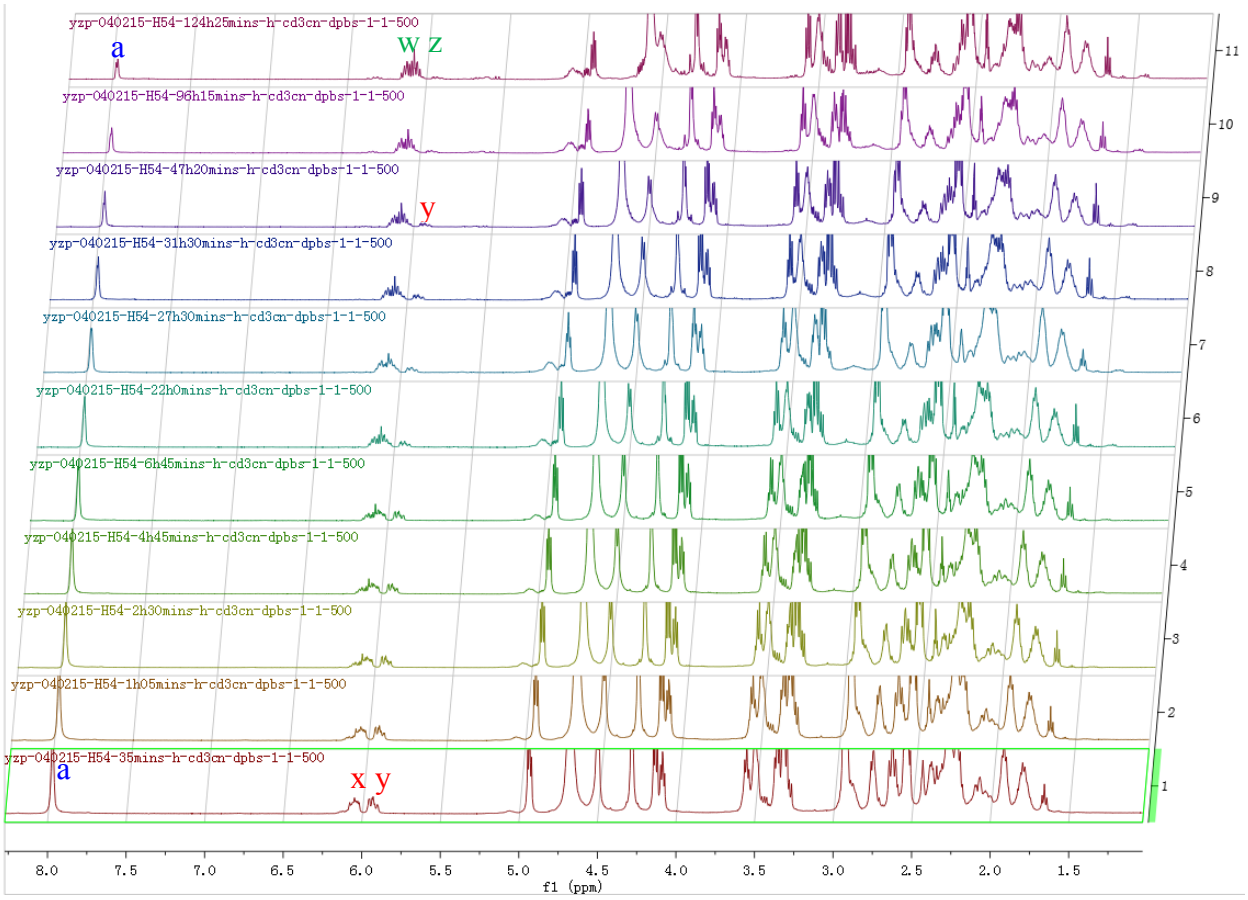


trans-cyclooctene (TCO; 20 mM)

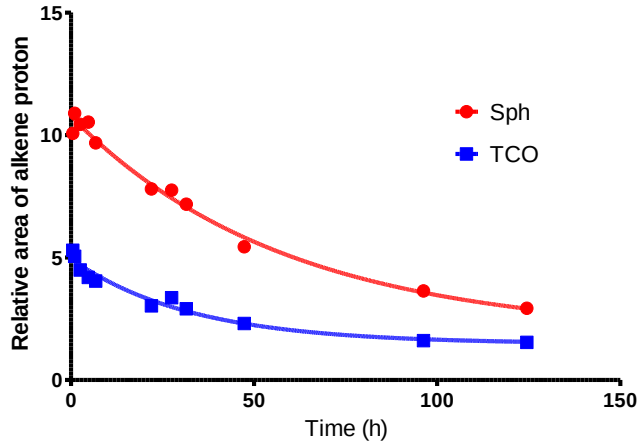


cis-cyclooctene (CCO)

b)



c)



Time (h)	Sph	TCO
0.50	10.07	5.30
1.00	10.89	5.05
2.50	10.44	4.49
4.75	10.53	4.19
6.75	9.68	4.04
22.00	7.80	3.03
27.50	7.75	3.36
31.50	7.18	2.91
47.30	5.44	2.31
96.25	3.64	1.61
124.50	2.93	1.54

d)

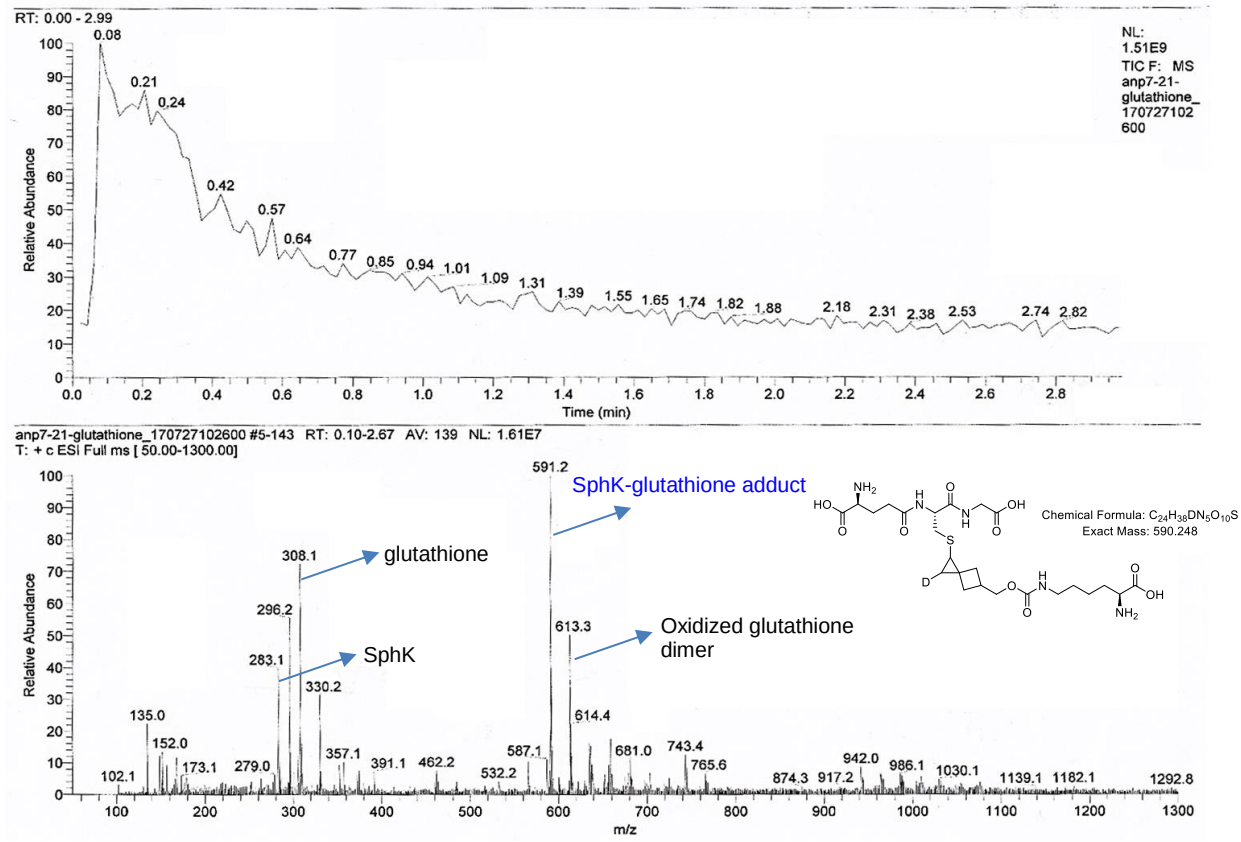


Figure S3. Stability comparison of spirohexene-L-lysine (SphK) vs. (*E*)-cyclooct-4-en-1-yl (3-aminopropyl)carbamate hydrochloride salt (TCO-amine•HCl salt) towards glutathione in mixed CD_3CN/deuterated PBS buffer (1:1) under argon as monitored by ^1H NMR. (a) Compounds used in the study. Incubation condition: 20 mM SphK and 20 mM TCO amine•HCl salt were mixed with 50 mM GSH (reduced form) in the deuterated solvent. (b) Time-lapsed NMR spectra of the reaction mixture. (c) Plot of intact strained alkenes in the mixture based on their characteristic ^1H signals. Integration of peak *a* was used in quantifying intact SphK while integration of peak *y* was used in quantifying intact TCO. The integration areas were listed in the table. The plots were fitted to the one-phase decay equation in GraphPad Prism 5: Y = (Y0 -

Plateau)*exp(-K*X) + Plateau, to give K_{Sph} = 0.0171 h^{-1} and K_{TCO} = 0.0304 h^{-1}. The half-life, $t_{1/2}$, was calculated based on the equation: $t_{1/2} = 0.693/K$, to give $t_{1/2, Sph}$ = 41 h and $t_{1/2, TCO}$ = 23 h. (d) LC-MS analysis of the mixture after incubating 20 mM SphK with 50 mM glutathione in CD_3CN/deuterated PBS buffer (1:1) at room temperature for 163 h. A SphK-glutathione adduct with a mass of 591.24 Da was detected. The plausible adduct structure was shown.

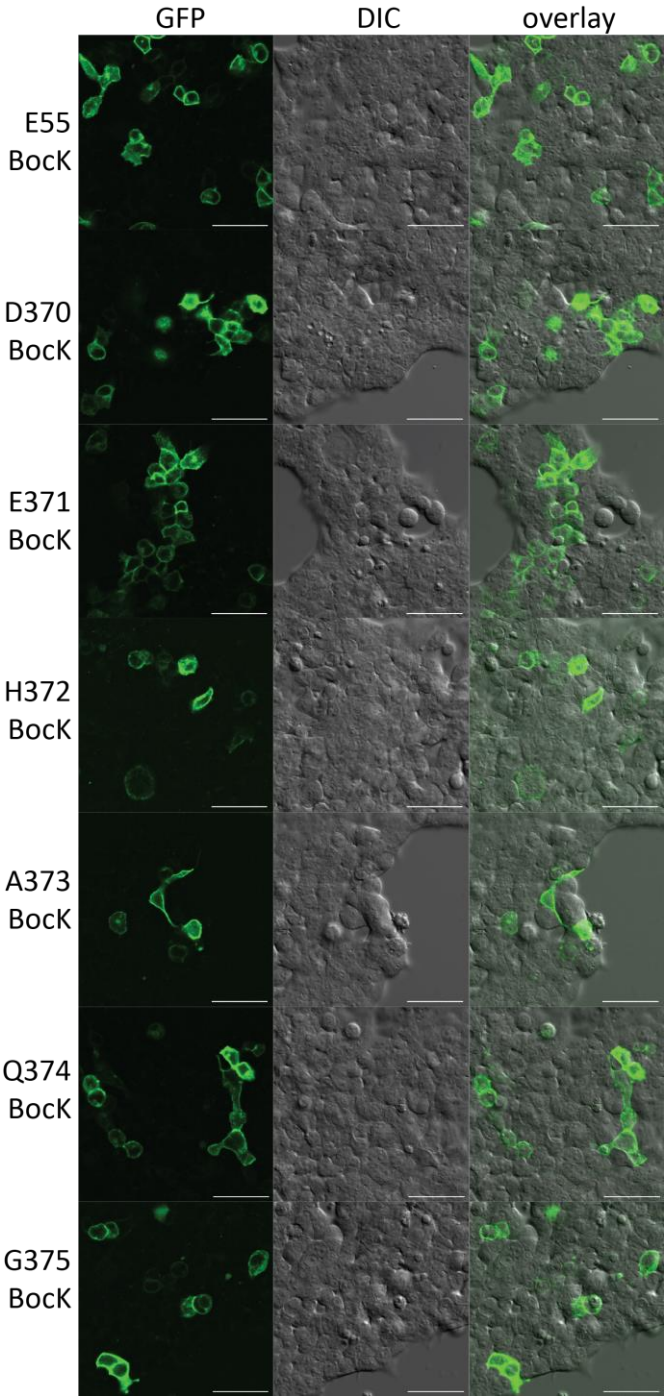


Figure S4. Incorporation of BocK into GCGR-GFP via amber codon suppression. Scale bar = 50 μm.

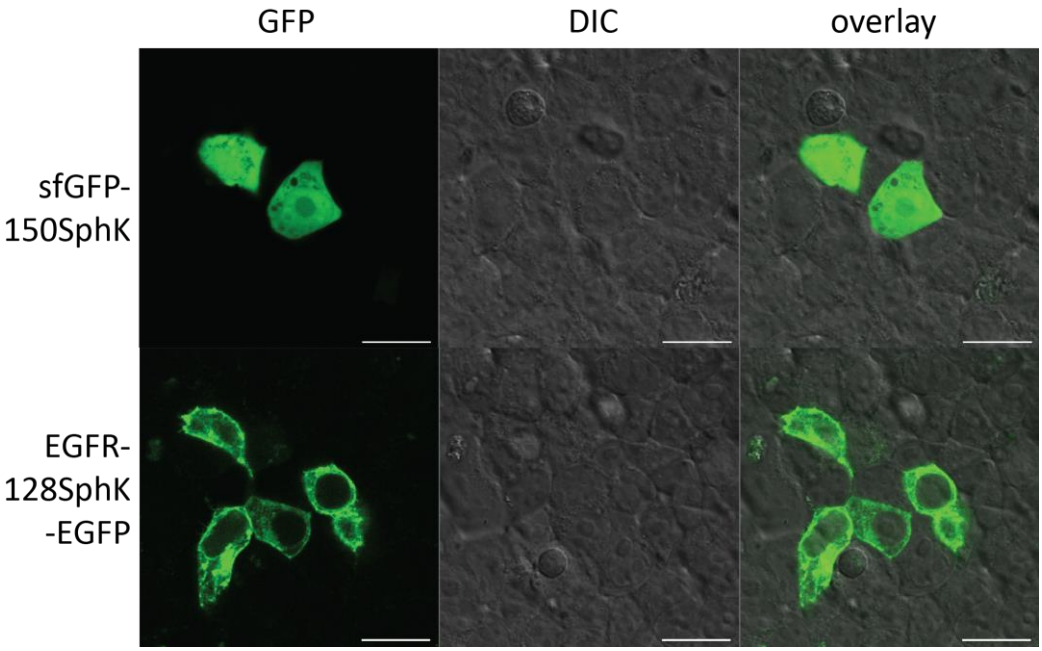


Figure S5. Validation of the SphK incorporation into the superfolder green fluorescent protein (sfGFP) and epidermal growth factor receptor (EGFR)-EGFP fusion proteins in HEK293T cells. Scale bar = 50 μm.

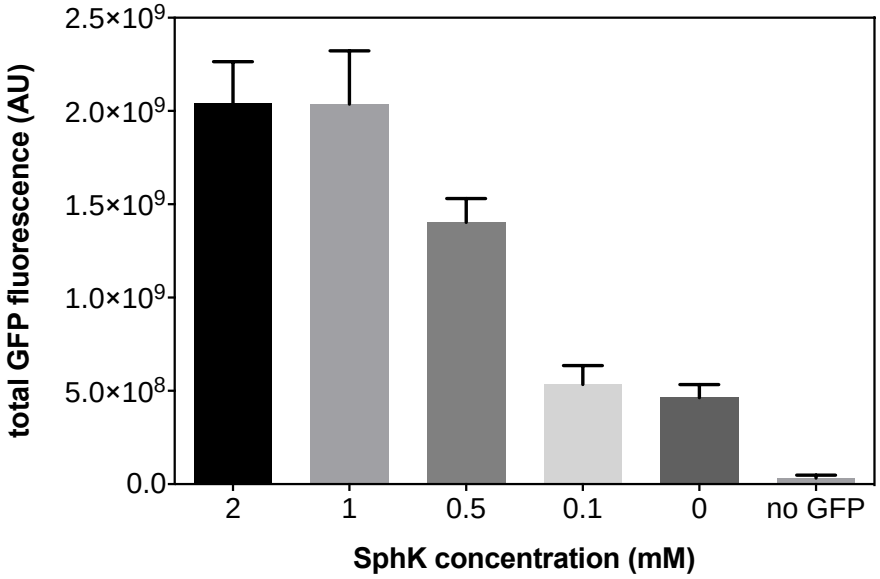


Figure S6. Effect of SphK concentration on the expression of sfGFP-150SphK through amber codon suppression in HEK293T cells.

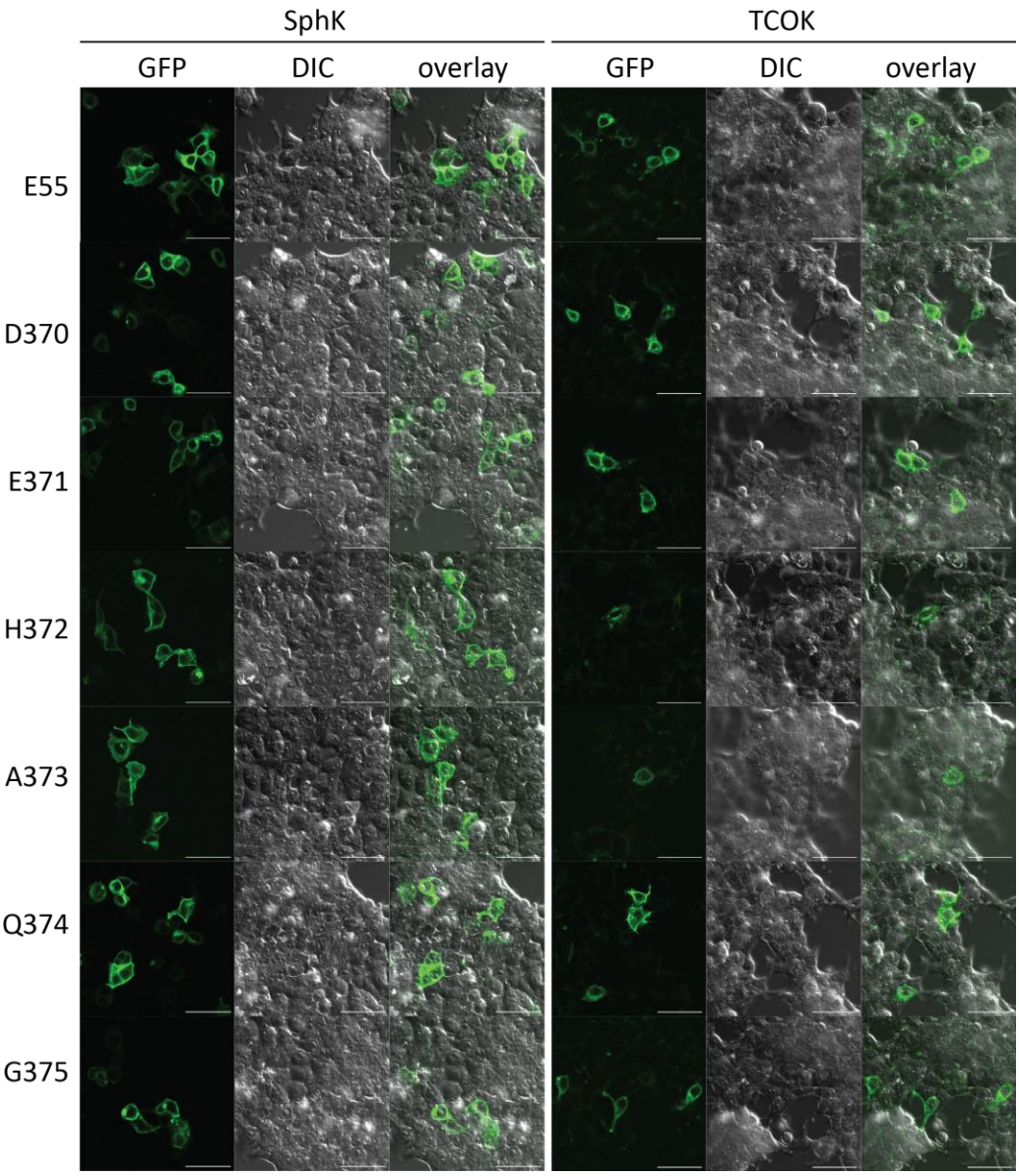


Figure S7. Incorporation of SphK and TCOK into the ECD and TMD domains of GCGR-GFP in HEK293T cells. Scale bar = 50 μm.

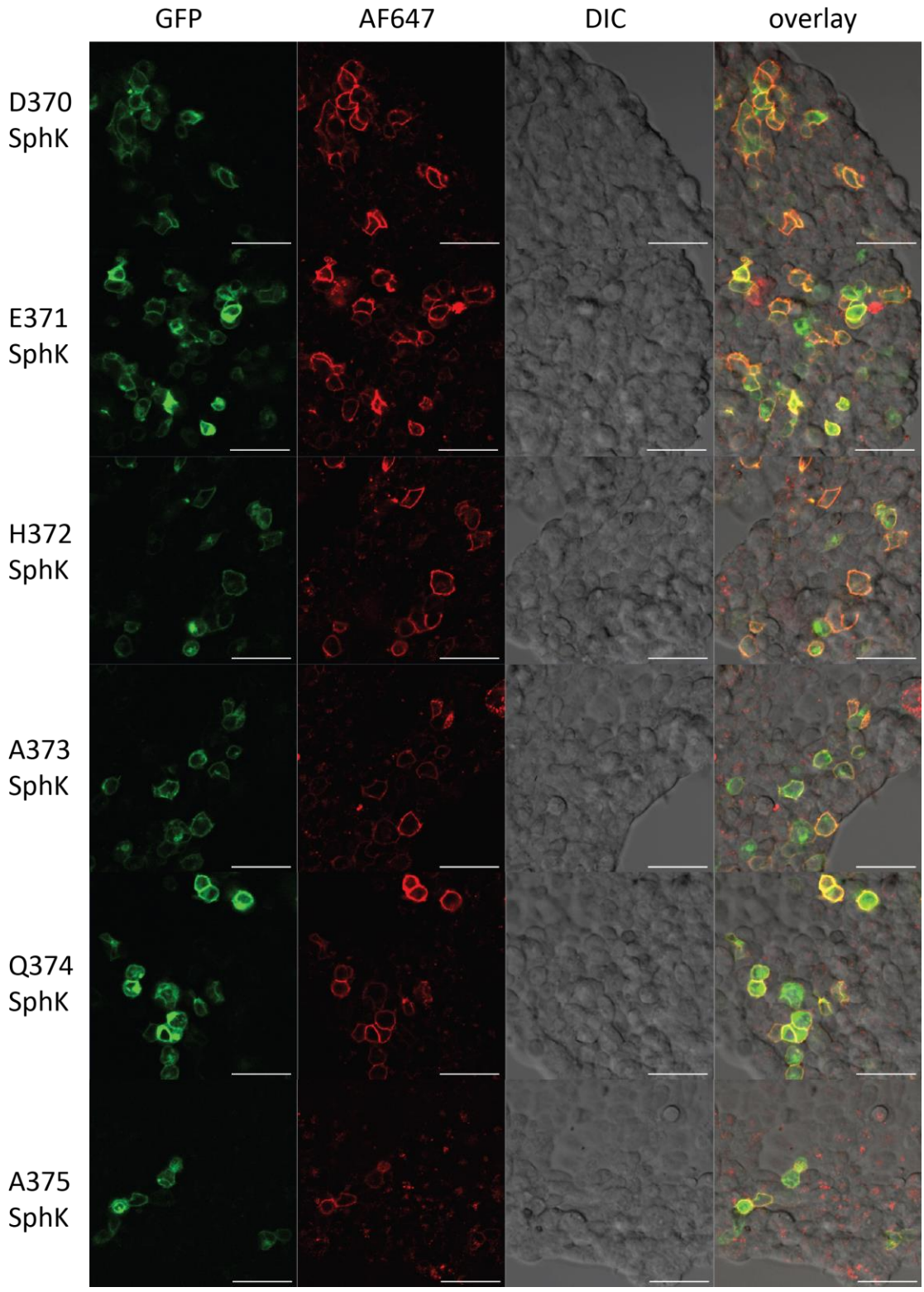


Figure S8. Bioorthogonal labeling of GCGR-GFP encoding SphK at various positions of the third extracellular loop in HEK293T cells. Scale bar = 50 μm.

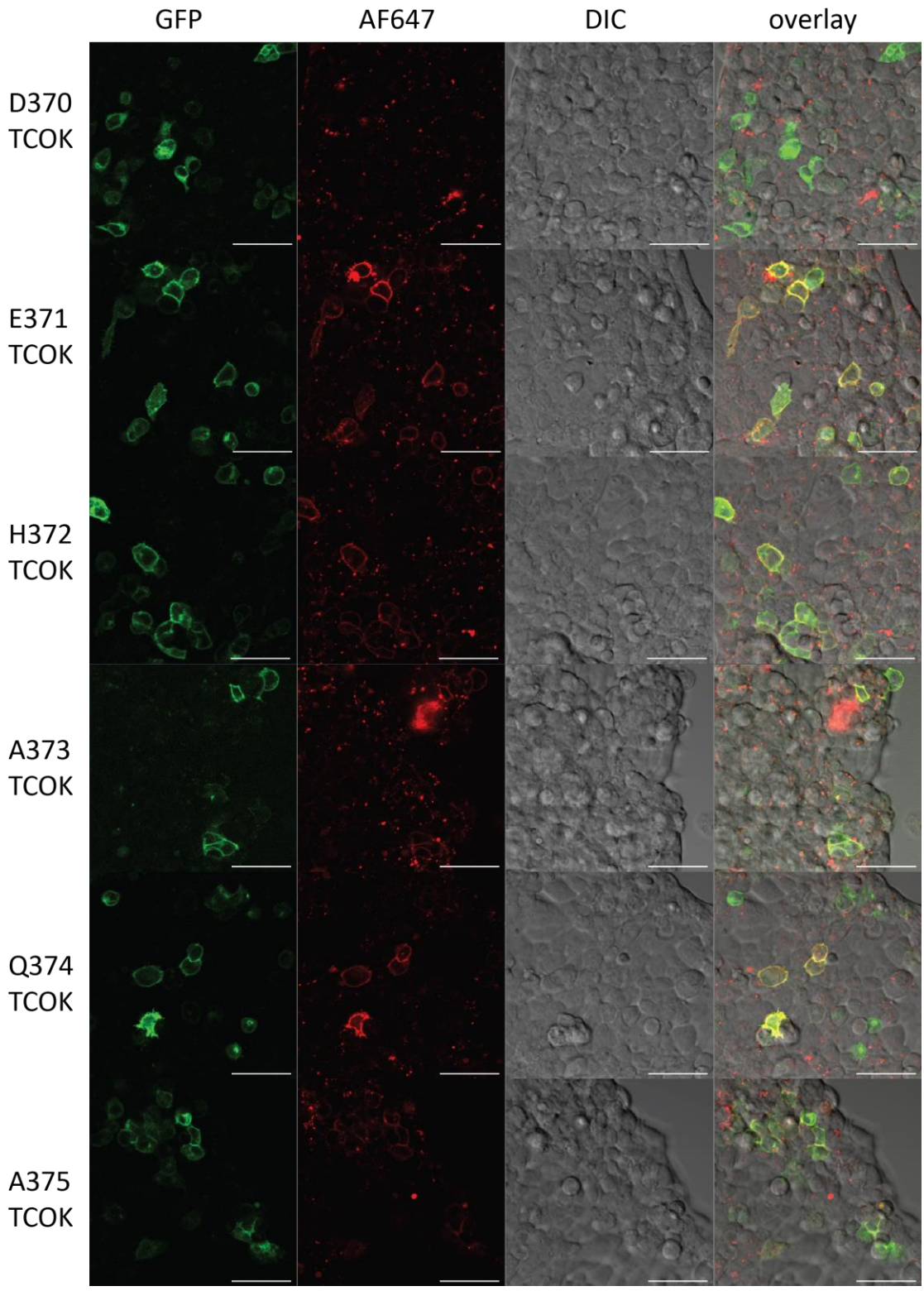


Figure S9. Bioorthogonal labeling of GCGR-GFP with TCOK at different positions on the third extracellular loop in HEK293T cells. Scale bar = 50 μm.

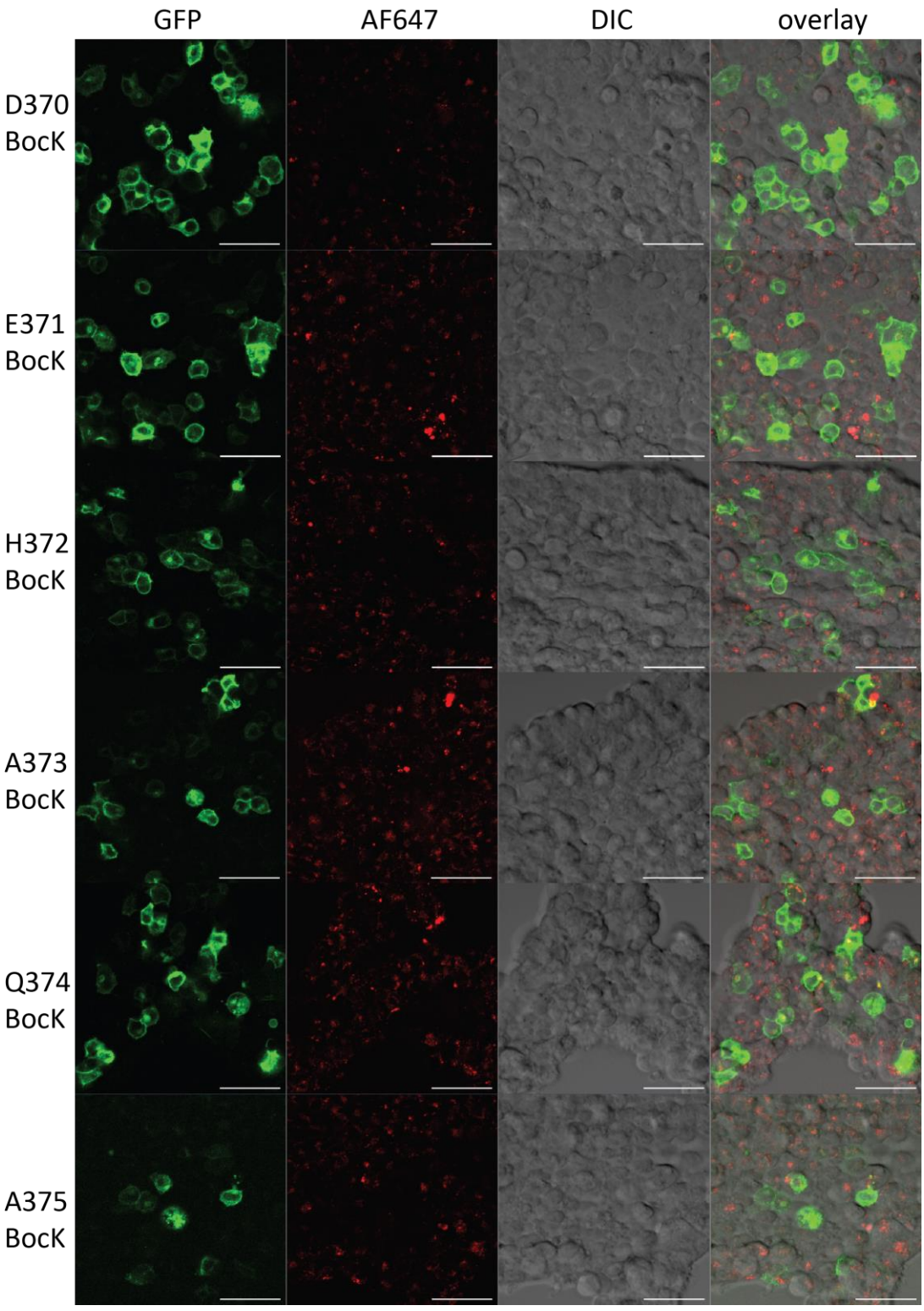


Figure S10. Control confocal micrographic images of HEK293T cells expressing the BocK-encoded GCGR-GFP mutants after treatment with 5 μM of DpTz-AF647 for 1 hour. Scale bar = 50 μm.

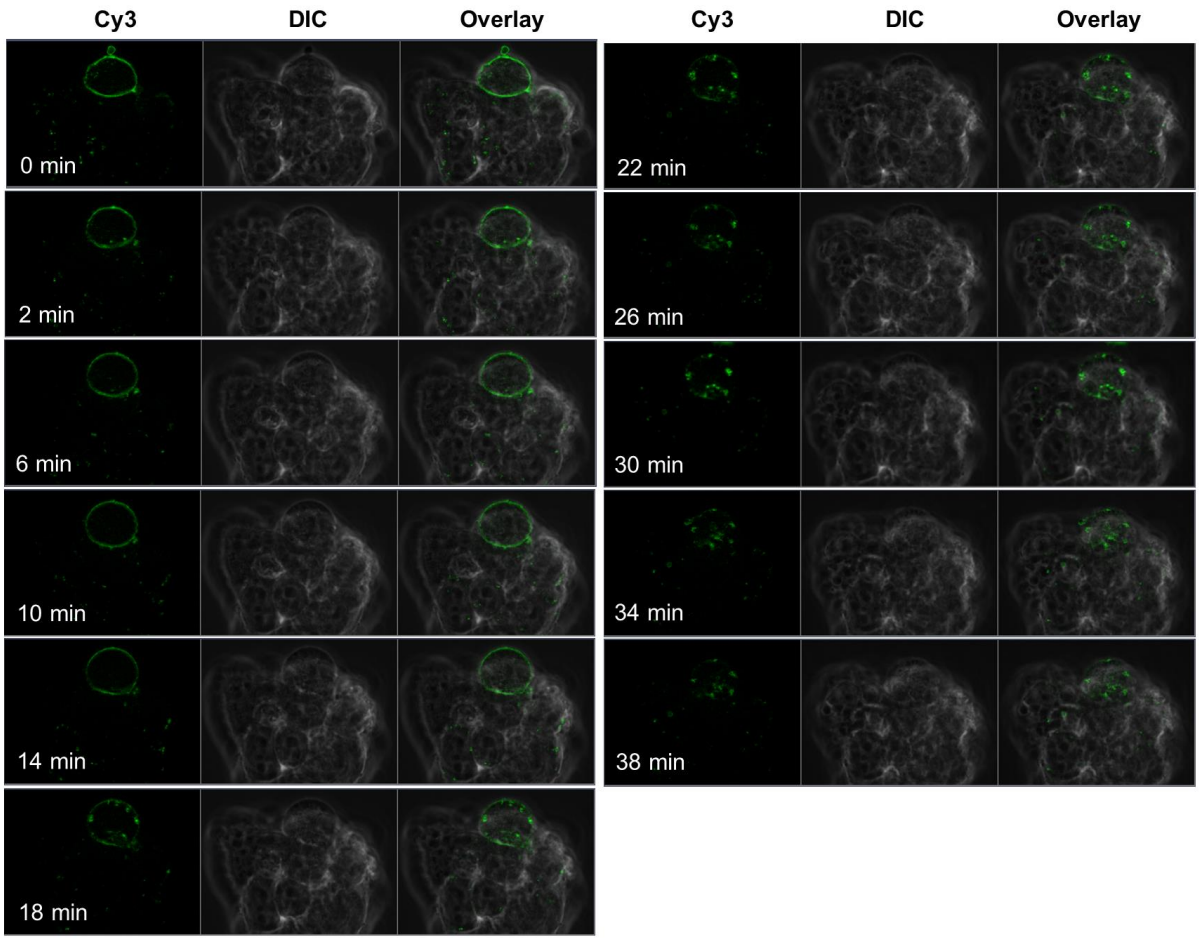


Figure S11. Time-lapsed fluorescent images of HEK293T cells showing gradual internalization of the Cy3-labeled GCGR-H372 mutant upon stimulation with 10 μM glucagon.

Table S1. Effect of unnatural amino acid mutagenesis of glucagon receptor (GCGR) response in glucagon-induced intracellular cAMP accumulation. pEC_{50} values represent the negative logarithm of the concentration of glucagon that produces half of the maximal response, E_{max}. E_{max} is presented as % of the WT response. Values are from a single experiment performed in duplicate.

GCGR mutant	pEC_{50}	E_{max}	GCGR mutant	pEC_{50}	E_{max}
WT	10.9	100	GCGR-GFP	11.1	66
E55BocK	10.2	93	E55TAG	9.7	11
D370BocK	10.9	3	D370TAG	8.7	2
E371BocK	7.4	79	E371TAG	8.7	2
H372BocK	**9.9**	**65**	**H372TAG**	**10.1**	**10**
A373BocK	9.5	5	A373TAG	6.2	8
Q374BocK	9.5	57	Q374TAG	9.4	13
G375BocK	8.3	54	G375TAG	10.6	4

Table S2. Effect of unnatural amino acid mutagenesis of GLP-1R response in GLP-1-induced intracellular cAMP accumulation. pEC_{50} values represent the negative logarithm of the concentration of glucagon that produces half of the maximal response, E_{max}. E_{max} is presented as % of the WT response. Values are from a single experiment performed in duplicate.

GLP-1R variant	pEC_{50}	E_{max}	GLP-1R variant	pEC_{50}	E_{max}
WT	10.0	100	GLP-1R-GFP	9.7	88
D59BocK	9.6	8	D59TAG	-	<1
E294BocK	-	<1	E294TAG	7.9	<1
G295BocK	9.8	4	G295TAG	-	<1
T298BocK	9.3	4	T298TAG	9.1	<1
S301BocK	10.2	3	S301TAG	9.7	1
E373BocK	8.0	3	E373TAG	9.4	1
H374BocK	**9.8**	**30**	**H374TAG**	**9.6**	**1**
A375BocK	7.4	4	A375TAG	8.2	1
R376BocK	9.5	5	R376TAG	9.5	1
G377BocK	**10.4**	**22**	**G377TAG**	**8.7**	**3**
T378BocK	**10.5**	**18**	**T378TAG**	**12.9**	**<1**

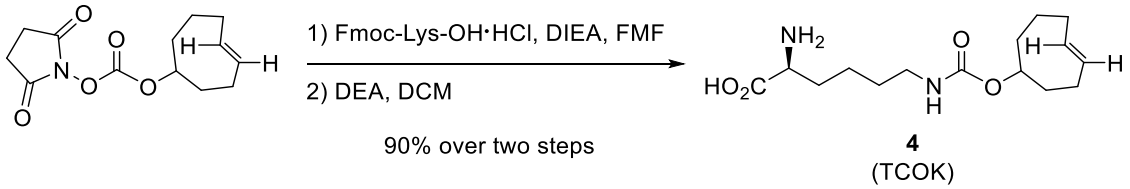
Table S3. Oligonucleotides used for amber mutations of glucagon and glucagon-like peptide 1 receptors.

Amino acid position	Primer sequence
GCGR	
E55	F: 5'- AGCCTGCTGCCCCCTCCCACGTAGCTGGTGTGCAACAGAACCTTCG -3'
	R: 5'- CGAAGGTTCTGTTGCACACCAGCTACGTGGGAGGGGGCAGCAGGCT -3'
D370	F: 5'- TTC GTG ACG TAG GAG CAC GCC CAG GGC ACC CTG -3'
	R: 5'- GGC GTG CTC CTA CGT CAC GAA GGC GAA GAC CAC -3'
E371	F: 5'- GTG ACG GAC TAG CAC GCC CAG GGC ACC CTG CGC -3'
	R: 5'- CTG GGC GTG CTA GTC CGT CAC GAA GGC GAA GAC -3'
H372	F: 5'- ACG GAC GAG TAG GCC CAG GGC ACC CTG CGC TCC -3'
	R: 5'- GCC CTG GGC CTA CTC GTC CGT CAC GAA GGC GAA -3'
A373	F: 5'- GAC GAG CAC TAG CAG GGC ACC CTG CGC TCC GCC -3'
	R: 5'- GGT GCC CTG CTA GTG CTC GTC CGT CAC GAA GGC -3'
Q374	F: 5'- GAG CAC GCC TAG GGC ACC CTG CGC TCC GCC AAG -3'
	R: 5'- CAG GGT GCC CTA GGC GTG CTC GTC CGT CAC GAA -3'
G375	F: 5'- CAC GCC CAG TAG ACC CTG CGC TCC GCC AAG CTC -3'
	R: 5'- GCG CAG GGT CTA CTG GGC GTG CTC GTC CGT CAC -3'
GLP-1R	
D59	F: 5'- GAGGATCCACCTCCTCCTGCCACATAGTTGTTCTGCAACCGGACCTTC G -3'
	R: 5'- CGAAGGTCCGGTTGCAGAACAACTATGTGGCAGGAGGAGGTGGATCCTC -3'
E294	F: 5'- GTCAAGTACCTCTATGAGGACTAGGGCTGCTGGACCAGGAACTCC -3'
	R: 5'- GGAGTTCCTGGTCCAGCAGCCCTAGTCCTCATAGAGGTACTTGAC -3'
G295	F: 5'- GAGGACGAGTAGTGCTGGACCAGGAACTCCAACATG -3'
	R: 5'- GGTCCAGCACTACTCGTCCTCATAGAGGTACTTGAC -3'
T298	F: 5'- GGCTGCTGGTAGAGGAACTCCAACATGAACTACTGG -3'
	R: 5'- GGAGTTCCTCTACCAGCAGCCCTCGTCCTCATAGAG -3'
S301	F: 5'- ACCAGGAACTAGAACATGAACTACTGGCTCATTATC -3'
	R: 5'- GTTCATGTTCTAGTTCCTGGTCCAGCAGCCCTCGTC -3'
E373	F: 5'- GTGATGGACTAGCACGCCCGGGGGACCCTGCGCTTC -3'
	R: 5'- CCGGGCGTGCTAGTCCATCACAAAGGCAAAGATGAC -3'
H374	F: 5'- TTTGCCTTTGTGATGGACGAGTAGGCCCGGGGGACCCTGCGCTTC -3'
	R: 5'- GAAGCGCAGGGTCCCCCGGGCCTACTCGTCCATCACAAAGGCAAA -3'
A375	F: 5'- GACGAGCACTAGCGGGGGACCCTGCGCTTCATCAAG -3'
	R: 5'- GGTCCCCCGCTAGTGCTCGTCCATCACAAAGGCAAAG -3'
R376	F: 5'- GAGCACGCCTAGGGGACCCTGCGCTTCATCAAGCTG -3'
	R: 5'- CAGGGTCCCCTAGGCGTGCTCGTCCATCACAAAGGC -3'
G377	F: 5'- CACGCCCGGTAGACCCTGCGCTTCATCAAGCTGTTTAC -3'
	R: 5'- GCGCAGGGTCTACCGGGCGTGCTCGTCCATCACAAAG -3'
T378	F: 5'- GCCCGGGGGTAGCTGCGCTTCATCAAGCTGTTTACAG -3'
	R: 5'- GAAGCGCAGCTACCCCCGGGCGTGCTCGTCCATCAC -3'

General Information

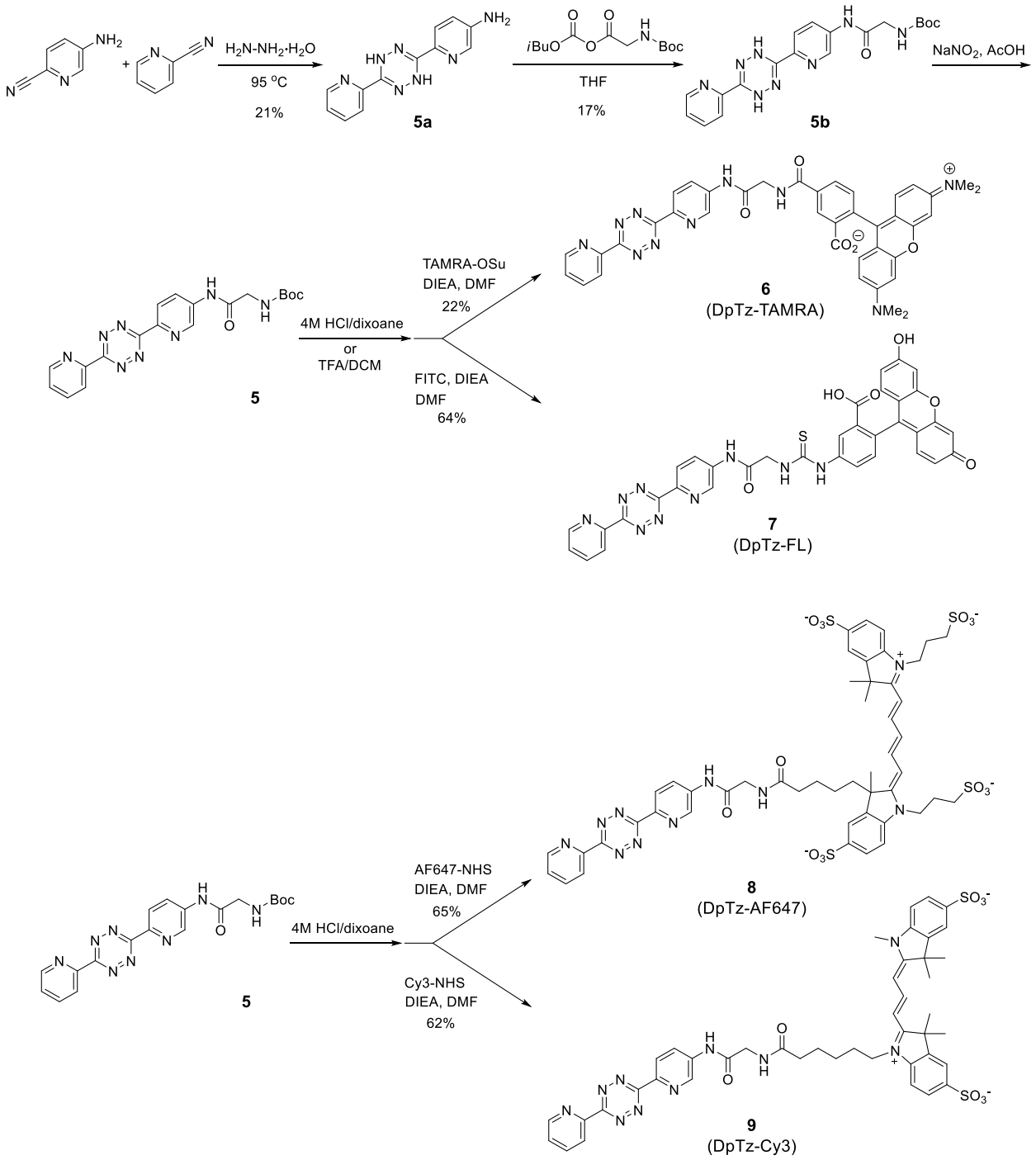
Solvents and chemicals were purchased from commercial sources and used directly without further purification. TCO (**2**) was purchased from Click Chemistry Tools (Cat. No. 1021). BG-amine was purchased from Oxchem (Cat. No. AX8216524). BocK was purchased from Chem-Impex (Cat. No. 00363). Flash chromatography was performed either manually with SiliCycle P60 silica gel (40-63 μm, 60 Å) or using an automatic Yamazen AKROS flash system equipped with SiliaSep HP pre-packed columns. ^{1}H NMR spectra were recorded with Inova-300, -400 or -500 MHz spectrometers. Chemical shifts were reported in ppm using TMS or deuterated solvents as internal standards (TMS, 0.00; $CDCl_3$, 7.26; CD_3OD, 3.31; DMSO-d_6, 2.50). Multiplicity was reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, brs = broad. ^{13}C NMR spectra were recorded at 75.4 MHz, and chemical shifts were reported in ppm using deuterated solvents as internal standards ($CDCl_3$, 77.0; DMSO-d_6, 39.5; CD_3OD, 49.05). UV-Vis absorption spectra were recorded using 1-cm quartz cuvettes on a HP-8452 Diode Array Spectrophotometer. Fluorescence spectra were recorded using 1-cm quartz cuvette on a Horiba FluoroMax-4 spectrofluorometer at 25 ºC. High resolution mass spectrometry was performed on a Bruker solariX XR Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS). Low resolution mass spectrometry was performed on a Thermo Finnigan LCQ Advantage mass spectrometer.

Experimental Procedures and Characterization Data



(*E*)-*N*$^{\varepsilon}$-((cyclooct-4-en-1-yloxy)carbonyl)lysine (TCOK; 4): To a solution of Fmoc-Lys-OH hydrochloric acid salt (75 mg, 0.187 mmol) in anhydrous DMF was added DIEA (82 μL, 0.47 mmol), and the resulting mixture was stirred at room temperature for 10 min. TCO-NHS ester (Click Chemistry Tools, Cat. No. 1016; 25 mg, 0.094 mmol) was then added, and the mixture was stirred at room temperature under argon for 8 hours. Afterwards, the solvent was removed under reduced pressure and the residue was diluted in saturated citric acid before extraction with ethyl acetate. The organic layers were combined, washed twice with water and brine, dried over anhydrous Na_2SO_4, and concentrated to dryness in vacuum. The residue was purified by silica gel flash chromatography to give the crude Fmoc-TCOK, which was used without further purification. Fmoc-TCOK was dissolved in 5 mL DCM and treated with 5 mL diethyl amine. After 8 hours, the mixture was concentrated under reduced pressure. The resulting residue was resuspended in 10 mL DCM/diethyl ether (1:1), filtered, washed with DCM (10 mL × 2), diethyl ether (10 mL × 2), and dried to give the titled product as a white solid (25 mg, 90% yield): ^{1}H NMR (300 MHz, D_2O) δ 5.58 (m, 1H), 5.42 (m, 1H), 4.13 (m, 1H), 3.58 (t, J = 7.5 Hz, 1H), 2.95 (t, J = 7.5 Hz, 2H), 2.23-1.20 (br, 16H); LRMS (ESI) calcd for $C_{15}H_{26}N_2O_4$ 299.2 [M+H$^+$], found 299.3.

Scheme S1. Synthesis of tetrazine reagents **5-9**.



6-(6-(Pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-amine (5a): Dihydrotetrazine **5a** was synthesized as an orange crystalline solid according to the literature procedure[S1] from the commercially available 5-aminopicolinonitrile (1.023 g, 21% yield): ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.70 (s, 1H), 8.65 (s, 1H), 8.61 (d, J = 4.5 Hz, 1H), 7.98-7.90 (m, 3H), 7.64 (d, J = 8.5 Hz, 1H), 7.53-7.50 (m, 1H), 6.99 (dd, J = 8.5, 2.5, Hz, 1H), 5.88 (s, 2H).

***tert*-Butyl (2-oxo-2-((6-(6-(pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)ethyl)carbamate (5b):** Dihydrotetrazine **5b** was obtained as an orange crystalline solid according to the literature procedure[S1] using **5a** (472.3 mg, 1.87 mmol) as the starting material (130 mg, 17% yield): ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.42 (s, 1H), 8.94 (s, 1H), 8.88 (s, 1H), 8.81 (d, J = 2.0 Hz, 1H), 8.64 (d, J = 4.5 Hz, 1H), 8.15 (dd, J = 10.0, 2.0 Hz, 1H), 7.98-7.91 (m, 3H), 7.54-7.52 (m, 1H), 7.14 (t, J = 6.0 Hz, 1H), 3.78 (d, J = 6.0 Hz, 2H), 1.40 (s, 9H).

***tert*-Butyl (2-oxo-2-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)ethyl)carbamate (5):** Dipyridinyl-tetrazine **5** was obtained as a purple red crystalline solid according to the literature procedure[S1] using $NaNO_2$ (41 mg, 0.585 mmol) as oxidant in AcOH (2 mL) at 0 °C. The desired product was purified by flash chromatography (73 mg, 61% yield): ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.64 (s, 1H), 9.05 (d, J = 2.0 Hz, 1H), 8.94 (d, J = 4.5 Hz, 1H), 8.63 (d, J = 8.5 Hz, 1H), 8.59 (d, J = 7.5 Hz, 1H), 8.42 (dd, J = 8.5, 2.5 Hz, 1H), 8.15 (td, J = 8.0, 2.0 Hz, 1H), 7.73 (dd, J = 7.5, 5.0 Hz, 1H), 7.18 (t, J = 6.0 Hz, 1H), 3.84 (d, J = 6.0 Hz, 2H), 1.42 (s, 9H).

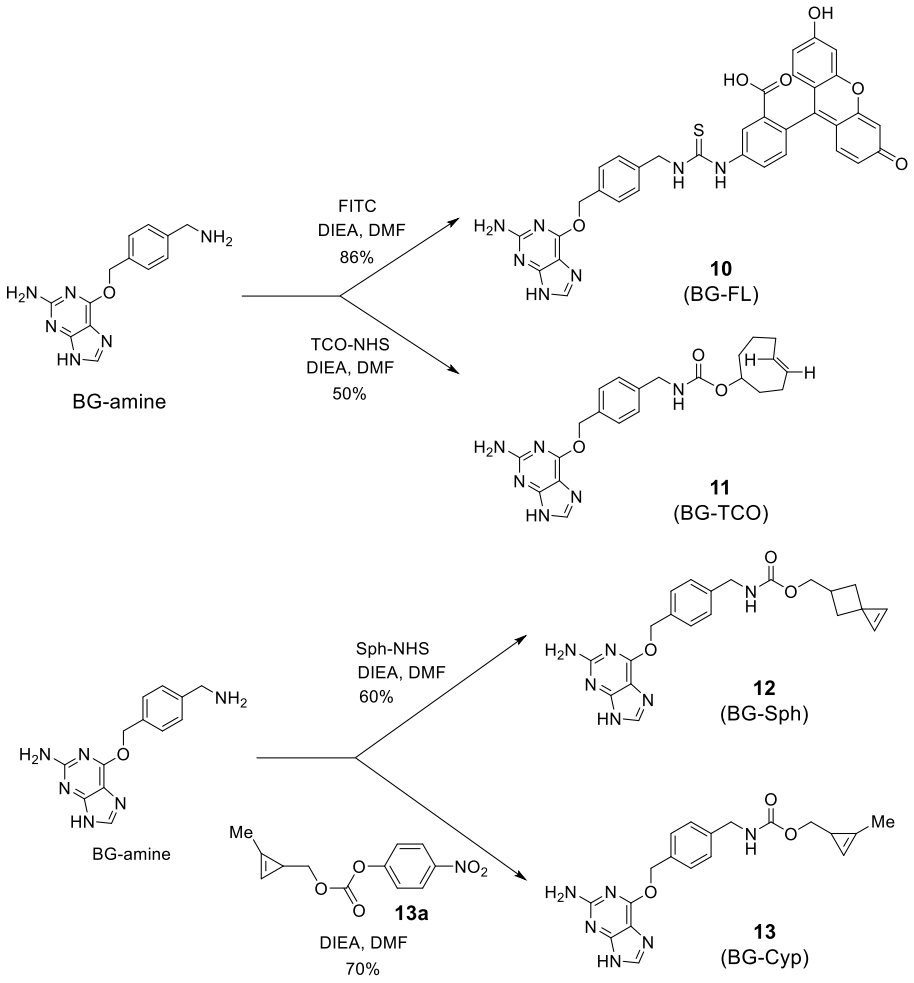
2-(6-(Dimethylamino)-3-(dimethyliminio)-3*H*-xanthen-9-yl)-5-((2-oxo-2-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)ethyl)carbamoyl)benzoate (DpTz-TAMRA; 6): To a solution of Boc-protected dipyridinyl-tetrazine **5** (15 mg, 0.037 mmol) in 1 mL DCM cooled in an ice-water bath was added 0.5 mL 4 N HCl in dioxane, and the mixture was stirred for 1 h. Then, DCM and HCl in dioxane were removed under reduced pressure to afford a red solid, which was used directly in subsequent steps without further purification: LRMS (ESI) calcd for $C_{14}H_{13}N_8O$ 309.1 [M+H$^+$], found 309.1. The solid was dissolved in 1 mL anhydrous DMF. To the resulting solution were added DIPEA (10 µL, 0.06 mmol), TAMRA-NHS ester (10 mg, 0.019 mmol) and the mixture was stirred at room temperature for 8 hours. The solvent was removed under reduced pressure and the residue was purified by silica gel flash chromatography using DCM and methanol as eluent to give the titled product as a dark red solid (3.0 mg, 22% yield, single isomer): LRMS (ESI) calcd for $C_{39}H_{32}N_{10}O_5$ 721.3 [M+H$^+$], found 721.2.

2-(6-Hydroxy-3-oxo-*3H*-xanthen-9-yl)-5-(3-(2-oxo-2-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)ethyl)thioureido)benzoate (DpTz-FL; 7): To a solution of Boc-protected dipyridinyl-tetrazine **5** (10 mg, 0.0245 mmol) in 0.25 mL DCM cooled in an ice-water bath was added 0.25 mL trifluoroacetic acid, and the mixture was stirred for 20 min. Then, DCM and TFA were removed under reduced pressure to afford a red solid, which was used directly in subsequent steps without further purification: LRMS (ESI) calcd for $C_{14}H_{13}N_8O$ 309.1 [M+H$^+$], found 309.1. The solid was dissolved in 0.5 mL anhydrous DMF. To the resulting solution were added DIPEA (22 µL, 0.122 mmol), FITC (9.53 mg, 0.0245 mmol) in several portions and the mixture was stirred at room temperature for 12 hours. The reaction was quenched by water and the product was dried by air flow. The residue was purified by silica gel flash chromatography using ethyl acetate/methanol (1:1, then 1:2) as eluent to give the titled product as a dark red solid (11.0 mg, 64% yield, single isomer): ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.96 (brs, 1H), 10.69 (brs, 1H), 10.29 (brs, 2H), 9.14 (d, J = 2.5 Hz, 1H), 8.95 (d, J = 4.5 Hz, 1H), 8.67 (d, J = 8.5 Hz, 1H), 8.61 (d, J = 7.5 Hz, 1H), 8.52-8.47 (brs, 1H), 8.47 (dd, J = 9.0, 2.5 Hz, 1H), 8.36 (s, 1H), 8.17 (td, J = 8.0, 2.0 Hz, 1H), 7.84 (dd, J = 8.5, 2.0 Hz, 1H), 7.74 (ddd, J = 7.5, 4.5, 1.0 Hz, 1H), 7.20 (d, J = 8.5 Hz, 1H), 6.70-6.48 (m, 6H), 4.53 (s, 2H); LRMS (ESI) calcd for $C_{35}H_{24}N_9O_6S$ 698.2 [M+H$^+$], found 698.1.

DpTz-AF647 (8) was synthesized according to a literature procedure.[S2]

DpTz-Cy3 (9) was synthesized according to a literature procedure.[S1] LRMS (ESI) calcd for $C_{44}H_{45}N_{10}O_8S_2$ 905.3 $[M-H]^-$, found 905.3.

Scheme S2. Synthesis of BG analogs **10-13**.



1-(4-(((2-Amino-9*H*-purin-6-yl)oxy)methyl)benzyl)-3-(3',6'-dihydroxy-3-oxo-3*H*-spiro[iso-benzofuran-1,9'-xanthen]-5-yl)thiourea (BG-FL; 10): Compound **10** was synthesized as a red-orange solid with 86% yield using the same procedure as compound **6**: LRMS (ESI) calcd for $C_{34}H_{26}N_7O_6S$ 660.2 $[M+H]^+$, found 660.1.

(*E*)-Cyclooct-4-en-1-yl (4-(((2-amino-9*H*-purin-6-yl)oxy)methyl)benzyl)carbamate (BG-TCO; 11) : To a solution of TCO-NHS ester (14 mg, 0.05 mmol) in DMF was added BG-amine (17 mg, 0.063 mmol) and DIEA (12 μL, 0.069 mmol), and the resulting mixture was stirred at room temperature overnight. The solvent was removed under vacuum, the residue was purified by silica gel chromatography using DCM and MeOH as eluent solvent to give the titled product as a white solid (11 mg, 50%): ^{1}H NMR (300 MHz, CD_3OD) δ 7.83 (s, 1H), 7.50 (d, J = 7.5, 2H), 7.29 (d, J = 7.5, 2H), 5.52 (s, 2H), 5.62-5.42 (brs, 2H), 4.36 (m, 1H), 4.28 (s, 2H), 2.38-1.56 (brs, 10H); HRMS (ESI-FT-ICR) calcd for $C_{22}H_{26}N_6O_3$ 423.2139 $[M+H^+]$, found 423.2140.

Spiro[2.3]hex-1-en-5-ylmethyl (4-(((2-amino-9*H*-purin-6-yl)oxy)methyl)benzyl)carbamate (BG-Sph; 12): BG-Sph was synthesized as a colorless solid with 60% yield using the same procedure as compound **11**: ^{1}H NMR (300 MHz, CD_3OD) δ 7.83 (s, 1H), 7.52 (s, 2H), 7.49 (d, J = 7.5, 2H), 7.29 (d, J = 7.5, 2H), 5.54 (s, 2H), 4.30 (s, 2H), 4.18 (d, J = 7.5, 2H), 2.50 (m, 1H), 2.21 (brs, 2H), 1.87 (brs, 2H); HRMS (ESI-FT-ICR) calcd for $C_{21}H_{22}N_6O_3$ 407.1826 [M+H$^+$], found 407.1838.

(2-Methylcycloprop-2-en-1-yl)methyl (4-nitrophenyl) carbonate (13a): The cyclopropene derivative **13a** was synthesized according to the reported procedure[S3]: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.28 (d, J = 10.0 Hz, 2H), 7.40 (d, J = 10.0 Hz, 2H), 6.61 (s, 1H), 4.21 (m, 1H), 4.16 (m, 1H), 2.19 (s, 3H), 1.78 (t, J = 2.0 Hz, 1H).

(2-Methylcycloprop-2-en-1-yl)methyl (4-(((2-amino-9*H*-purin-6-yl)oxy)methyl)benzyl) carbamate (BG-Cyp; 13): Compound **10** was synthesized using the same procedure as compound **11** as a white solid with 70% yield: ^{1}H NMR (300 MHz, CD_3OD) δ 7.82 (s, 1H), 7.48 (s, d, J = 7.5 Hz, 2H), 7.30 (d, J = 7.5 Hz, 2H), 6.61 (s, 1H), 5.52 (s, 2H), 4.26 (s, 2H), 3.96 (m, 1H), 3.82 (m, 1H), 2.09 (s, 2H), 0.62 (t, 1H); HRMS (ESI-FT-ICR) calcd for $C_{19}H_{20}N_6O_3$ 381.1670 [M+H]$^+$, found 381.1673.

Cloning and Mutagenesis

SNAP-tag. His-tag was inserted into the C-terminus of SNAP-tag using Q5 site-directed mutagenesis kit (NEB) and *pSNAP-tag(T7)-2* (NEB) as template to make *pSNAP-C-His* using the primer pair:
F: 5'- caccaccacTAAGCGGCCGCATTGATC -3'
R: 5'- atgatgatgTCCCAGACCCGGTTTACC -3'

SNAP-GLP-1R. The cloning sites *HindIII* and *NheI* were introduced by PCR into *pCMV6-GLP-1R-myc-DDK* (OriGene) immediately after the signal sequence to make *pCMV6-SP-HN-GLP-1R-myc-DDK* using the primer pair:
F: 5'- tcaactaggaccggctagcCGCCCCCAGGGTGCCACTG -3'
R: 5'- gatctggcgcgccgaagcttGGGGCCGGCCCTGCCCAC -3'
Mammalian codon-optimized SNAP-tag gene was custom synthesized (Genscript) and cloned into *pCMV6-SP-HN-GLP-1R-myc-DDK* at the N-terminus to make *pCMV6-SNAP-GLP-1R*.

SNAP-GCGR. The cloning sites *HindIII* and *NheI* were introduced by PCR into *pCMV6-GCGR-GFP* (OriGene) immediately after the signal sequence to make *pCMV6-SP-HN-GCGR-GFP* using the primer pair:
F: 5'- CAACTAGGACCGGCTAGCGCTCAGGTGATGGACTTCCTG -3'
R: 5'- ACTGGCGCGCCGAAGCTTGGAGGGGACCTGTGGCTG -3'

SNAP-tag gene was cloned into *pCMV6-SP-HN-GCGR-GFP* at the N-terminus to make *pCMV6-SNAP-GCGR-GFP*. SNAP-GCGR was digested using *SgfI* and *MluI* then cloned into *pCMV6-GLP-1R-myc-DDK* to make *pCMV6-SNAP-GCGR*.

TCOKRS. TCOKRS carries three mutations in active site: Y306A, L309M and C348A.[S4] The first round of mutation - Y306A/L309M – was carried out by PCR using the template *pCMV-MmPylRS-U6-PylT*[S5] and the primer pair:

F: 5'- CCCATGCTTGCTCCAAACCTTGCGAACTACATGCGCAAGCTTGACAGGGCCCTG -3'
R: 5'- CAGGGCCCTGTCAAGCTTGCGCATGTAGTTCGCAAGGTTTGGAGCAAGCATGGG -3'

A second round of PCR was performed to introduce the C348A mutation using the primer pair:
F: 5'- GAGTTTACCATGCTGAACTTCGCGCAGATGGGATCGGGATGCACA -3'
R: 5'- TGTGCATCCCGATCCCATCTGCGCGAAGTTCAGCATGGTAAACTC -3'

Amber mutation. Amber codon was introduced into selected positions in GCGR and GLP-1R by site-directed mutagenesis using Platinum Pfx DNA polymerase (Thermo Fisher Scientific) or Phusion high-fidelity DNA polymerase (New England Biolabs) following manufacturers' instructions using the primer pairs listed in Table S3 and one of the following templates: (1) *pCMV6-GCGR-GFP*, (2) *pcDNA3-GCGR-HA*, (3) *pCMV6-GLP-1R-GFP*, (4) *pCMV6-GLP-1R-myc-DDK*.

Reference:

[S1] Lang, K.; Davis, L.; Torres-Kolbus, J.; Chou, C.; Deiters, A.; Chin, J. W. *Nature Chem.* **2012**, *4*, 298-304.

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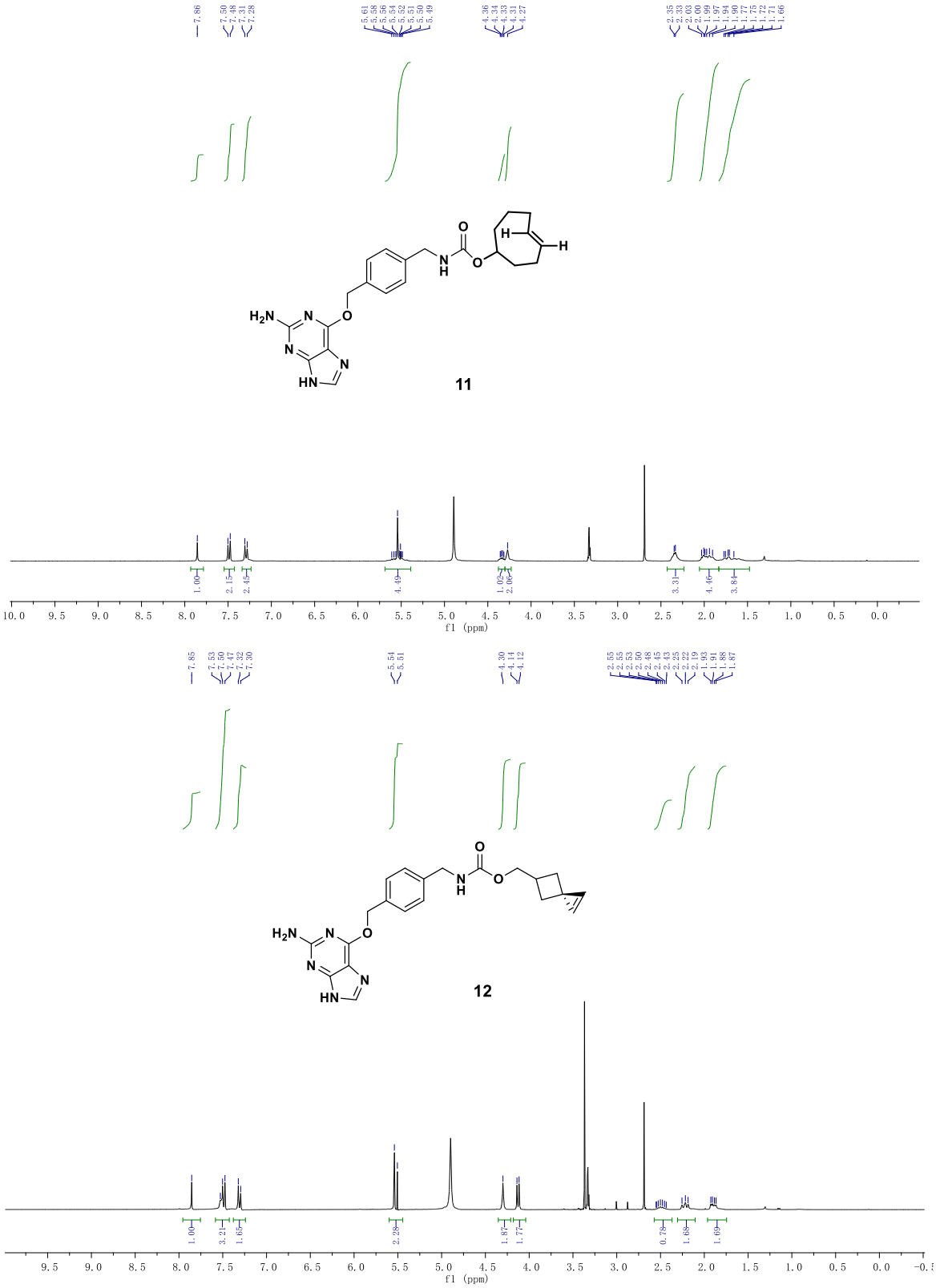
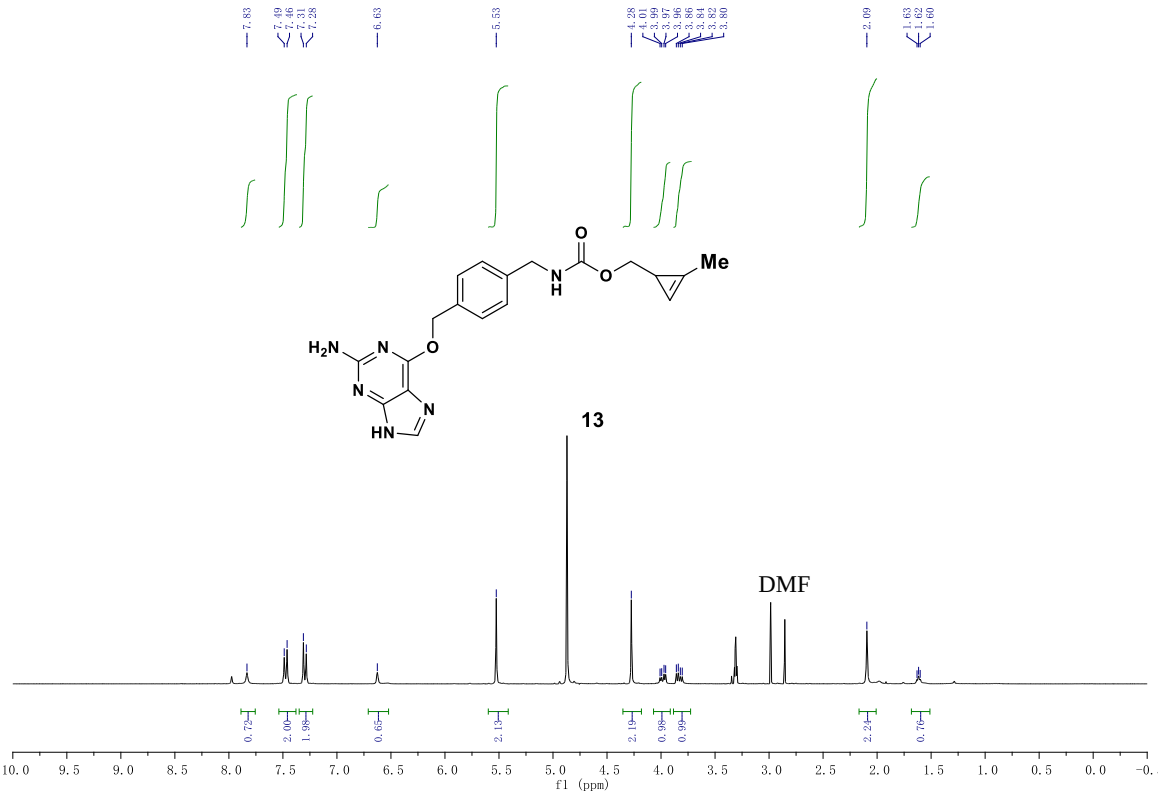
[S3] Patterson, D. M.; Nazarova, L. A.; Xie, B.; Kamber, D. N.; Prescher, J. A. *J. Am. Chem. Soc*. **2012**, *134*, 18638-18643.

[S4] Lang, K.; Davis, L.; Wallace, S.; Mahesh, M.; Cox, D. J.; Blackman, M. L.; Fox, J. M.; Chin, J. W. *J. Am. Chem. Soc*. **2012**, *134*, 10317-10320.

[S5] Li, N.; Ramil, C. P.; Lim, R. K. V.; Lin, Q. *ACS Chem. Biol.* **2015**, *10*, 379-384.

Spectral data

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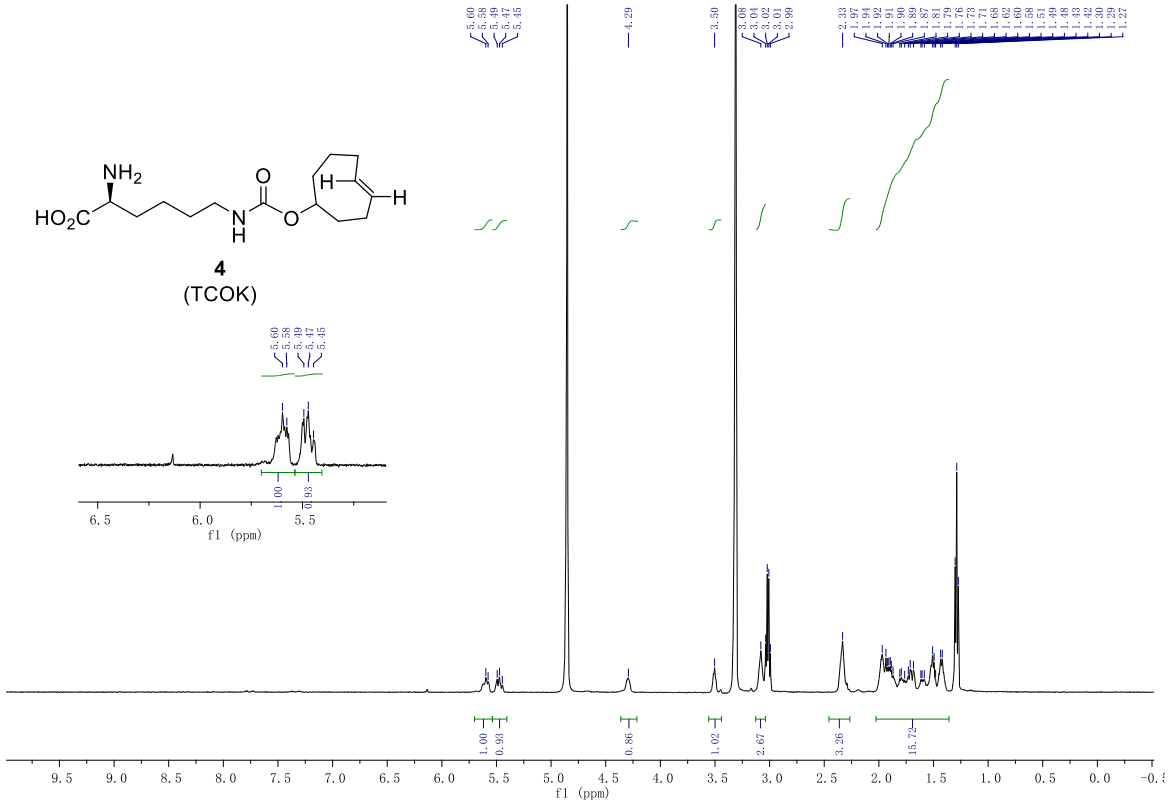
4
(TCOK)
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