Supplementary Information

Single-molecule FRET and conformational analysis of beta-arrestin-1 through genetic code expansion and Se-Click reaction

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Experimental Procedures

Materials and Reagents:
Cy5 was purchased from Lumiprobe, FlAsH-EDT_2 was purchased from Univ-bio (Shanghai, China). All other chemicals were purchased from Innochem (Beijing, China) and used without further purification. PCR reagents and restriction endonucleases were obtained from Fermentas. Glutathione-Sepharose 4B and Co–NTA affinity purification regents were purchased from Amersham Pharmacia. Anti-flag antibody M1 and flag beads were purchased from Sigma. Primers and genes were synthesized by Sangon Biotech. V2Rpp was obtained by the Tufts University core facility (Boston, USA). Anti-his antibody was purchased from Genscript.
^1H and ^{13}C NMR spectra were recorded on a Bruker AMX-500 instrument with chemical shifts reported relative to tetramethylsilane. Mass spectra of chemicals were analyzed on Waters LC-MS equipped with a single-quadrupole mass detector and an electrospray ionization source. Mass spectra of protein were run on an Angilent 6100 series of single quadrupole mass spectrometer (Agilent Technologies). Protein purification was performed at AKTA UPC 900 FPLC system (GE healthcare). Fluorescence spectra were recorded on a microplate reader equipped with SkanIt software 2.4.3 RE for Varioskan Flash (Varioskan Flash, Thermo Fisher Scientific Inc). Single molecule fluorescence resonance energy transfer (smFRET) measurement was performed on a home-built objective-type total internal reflection fluorescence (TIRF) microscope, based on a Nikon Eclipse Ti-E with an EMCCD camera (Andor iXon Ultra 897), and solid state 488 nm and 532 nm excitation lasers (Coherent Inc. OBIS Smart Lasers).
Genetic selection of the mutant synthetase specific for SeF: Plasmid pBK-lib-jw1 encodes a library of *M. jannaschii* tyrosyl tRNA synthetase (TyrRS) mutants randomized at residues Tyr32, Leu65, Phe108, Gln109, Asp158 and Leu162; and any one of the six residues (Ile63, Ala67, His70, Tyr114, Ile159, Val164) was either mutated to Gly or kept unchanged. Plasmid pREP(2)/YC encodes *Mj*$tRNA_{CUA}^{Tyr}$, the chloramphenicol acetyltransferase (CAT) gene with a TAG codon at residue 112, the GFP gene under control of the T7 promoter, and a Tet^r marker; plasmid pLWJ17B3 encodes *Mj*$tRNA_{CUA}^{Tyr}$ under the control of the *lpp* promoter and *rrnC* terminator, the barnase gene (with three amber codons at residues 2, 44 and 65) under the control of the *ara* promoter, and an Ampr marker. pBK-lib-jw1 consisting of 2×10^9 TyrRS independent clones was constructed using standard PCR methods. *E. coli* DH10B harboring the pREP(2)/YC plasmid was used as the host strain for the positive selection. Cells were transformed with the pBK-lib-jw1 library, recovered in SOC for 1 h, washed twice with glycerol minimal media with leucine (GMML) before plating on GMML-agar plates supplemented with kanamycin, chloramphenicol, tetracycline and SeF at 50 μg/mL, 60 μg/mL, 15 μg/mL and 1 mM respectively. Plates were incubated at 37℃ for 60 h and surviving cells were scraped and plasmid DNA was extracted and purified by gel electrophoresis. The pBK-lib-jw1 DNA was then transformed into electro-competent cells harboring the negative selection plasmid pLWJ17B3, recovered for 1 h in SOC and then plated on LB-agar plates containing 0.2% arabinose, 50 μg/mL ampicillin and 50 μg/mL kanamycin. The plates were then incubated at 37℃ for 8-12 h, and pBK-lib-jw1 DNA from the surviving clones was extracted as described above. The library was then carried through a subsequent round of positive selection, followed by a negative selection and a final round of positive selection (with chloramphenicol at 70 μg/mL). At this stage, 96 individual clones were selected and suspended in 50 μL of GMML in a 96-well plate, and replica-spotted on two sets of GMML plates. One set of GMML-agar plates was supplemented with tetracycline (15 μg/mL), kanamycin (50 μg/mL) and chloramphenicol at concentrations of 60, 80, 120 and 160 μg/mL with 1 mM SeF. The other set of plates were identical but did not contain SeF, and the chloramphenicol concentrations used were 0, 20, 40 and 60 μg/mL. After 60 h incubation at 37℃, one clone was found to survive at 160 μg/mL chloramphenicol in the presence of 1 mM SeF, but only at 20 μg/mL chloramphenicol in the absence SeF.
Synthetase expression and crystallization setup: DNA fragments encoding the SeFRS were amplified by PCR and cloned into the Nde Ⅰ and Xho Ⅰ sites of the expression vector pET22b, which was then transformed in BL21 (DE3) cells and grown to an OD of 0.8. After induction overnight with 1 mM IPTG, cells were pelleted by centrifugation and resuspended in lysis buffer (50 mM Tris, pH 8.5, 500 mM NaCl, 10 mM β-mercaptoethanol, 5 mM imidazole). Cells were sonicated, and the cell lysate was pelleted by centrifugation. The supernatant was collected and incubated with Ni- NTA agarose beads for 2 h at 4℃ , filtered, and washed with wash buffer (50 mM Tris, pH 8.5, 500 mM NaCl, 10 mM β-mercaptoethanol, 20 mM imidazole). The synthetase was eluted with a eluted buffer containing 300 mM imidazole in buffer A (25 mM Tris, pH 8.5, 25 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA), purified by anion exchange chromatography (Hitrap MonoQ; GE Healthcare) using a salt gradient from 25 mM to 0.5 M NaCl. SeFRS was purified by Sephadex gel column chromatography (Superdex 200, 10/300 GL; GE Healthcare) in a buffer containing 50 mM Tris, pH 8.5, 500 mM NaCl, 10 mM β-mercaptoethanol and concentrated to 20-25 mg/mL. Crystals of the mutant SeFRS were grown at 16℃ by using the handing drop vapor diffusion technique against a mother liquor composed of 18–25% polyethylene glycol (PEG) 3350, 100 mM Bis-Tris (pH 5.5) and 200 mM $MgCl_2$ and concentrated synthetase (25 mg/mL). The crystal appeared after about one week. Crystals were flash frozen in liquid nitrogen after a 30 s soak in 23% PEG 3350, 100 mM Bis-Tris (pH 5.5) 100 mM $MgCl_2$ and 20% ethylene glycol.
X-ray crystallography: X-ray diffraction data of SeFRS were collected at BL19U1 of Shanghai Synchrotron Radiation Facility (SSRF) at a single wavelength of 0.9789 Å. The data were reduced and scaled using the HKL3000 package. The structure of SeFRS complex was solved by molecular replacement using wild- type *M. jannaschii tyrosyl*-tRNA synthetase (PDB code: 1J1U) as a search model in which water molecules and other heteroatoms were deleted and processed by Molrep of the CCP4 package. Structural refinement was carried out by Refmac5 in the CCP4 program suite. In the refinement process, the program Coot in the CCP4 program suite was used for the model building (main chain tracing), ligand and water finding, and real space refinement of side chains and zones. The coordinate of SeFRS was genetrated by ProDrg and program Coot in the CCP4 program suite.
Molecular docking of SeF into SeFRS: Glide program 1,2 embedded in Maestro 7.5 was used to carry out molecular docking. First the coordinates of the protein originated from SeFRS were minimized using the Protein Preparation Wizard Workflow with default settings, then docking grids were created by defining residues within 15 Å around Tyr binding site. Finally, the ligand SeF prepared by the LigPrep panel (version 2.3, Schrödinger, LLC, New York, NY) were docked into the well-defined docking grids with the extra precision (XP) mode.
Trypsin digestion and LC-MS/MS analysis: The βarr1-T6SeF was subjected to SDS-PAGE. The protein band was cut into small fragments and washed twice in distilled water for 10 min. The protein bands were dehydrated in pure CH_3CN for 10 min and dried in

Speedvac for 15 min. Protein were reduced by DTT (10 mL, 100 mM) and alkylated by iodoacetamide (40 mM) for 45 min in dark at 25℃. Then, the sample was digested by trypsin with a ratio of 100:1 overnight. The digested fragments were purified, desalted and resolved in 30 mL 50% acetonitrile and 0.1% trifluoroacetic acid buffer before LC-MS/MS analysis.

Digested peptides analysis was performed using Thermo Finnigan LTQ linear ion trap mass spectrometer in line with a Thermo Finnigan Surveyor MS Pump Plus HPLC system. The trypsin digested peptides were loaded onto a trap column (C18, 15 cm × 75 μm, 3 μm particle) (Dr. Maisch GmbH, Ammerbuch), which was connected with the self-packed analytical column (C18, 3 cm × 15 μm, 3 μm particle) (Dr. Maisch GmbH, Ammerbuch). The peptides were eluted over a gradient at a flow rate of 300 nL/min for 90 min (A: 0.1 FA/H2O, B: 0.1% FA/ 80% CH_3CN/20% H_2O). MS data were analysed by Proteome Discoverer (version 1.4.0.288. Thermo Fischer Scientific) software. MS2 spectrum was retrieved by SEQUEST.

Expression and purification of CaM66SeF: For the expression of CaM66SeF, the plasmid pGEX-6p-1/CaM66TAG110TGA and pEVOL/SeFRS were co-transformed into BL21. A single colony was grown overnight at 37 °C in 4 mL LB medium and amplified in 100 mL LB medium to OD600 of 1.0. The protein expression was induced by 0.3 mM IPTG, 0.02% L-arabinose and 0.5 mM SeF in the presence of 0.5 mM TCEP. After growing for 4 h at 37℃, the cells were harvested, and resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl). After sonication and centrifugation, the supernatant was purified by Ni-NTA affinity chromatography to generate GST-CaM. GST-CaM was bound to GST beads and incubated with HRV-3C protease at 4℃ for 12 h. The GST cleaved CaM was eluted, concentrated and verified by SDS-PAGE electrophoresis.

Peptide synthesis：A fully phosphorylated 29 amino-acid carboxy-terminal peptides corresponding to the sequence of the C-terminal of the human V2 vasopressin receptor (V2Rpp:343ARGRpTPPpSLGPQDEpSCpTpTApSpSpSLAKDTSS371) was synthesized from Tufts University Core Facility as described previously.[1-4] GGG-V2Rpp (GGGARGRpTPPpSLGPQDEpSCpTpTApSpSpSLAKDTSS) was synthesized for sortase ligation reactions.[5]

Expression and purification of pp-β_2V2R:

β2-adrenergic receptor (β2AR) construct were of human origin and contained an N-terminal flag tag and a C-terminal 10xHis tag.[5] For β2AR used in sortase ligation, the sortase consensus site (LPETGG) was inserted after amino acid 365 (β2AR-LPETGG). β2AR construct was expressed in sf9 insect cells using the Bac-to-Bac baculovirus Expression System. Cells were infected at a density of 3 × 10^6 cells per milliliter and harvested 60 h thereafter. Cells were resuspended by hypotonic buffer C (10 mM HEPES pH 7.5, 20 mM KCl, 1 μM alprenolol, protease inhibitor) and homogenized using a Dounce homogenizer. The membrane fraction was separated via ultra-centrifugation (39,000 rpm*50 min) and washed 4 times with high osmotic buffer D (1.0 M NaCl in buffer C). Receptor was solubilized with buffer E (50 mM HEPES, pH 7.5, 1 M NaCl) containing 1% n-decyl-β-D-maltopyranoside (DDM, Anatrace) and 0.2% CHS (sigma), then purified by flag-M1 resin (sigma) affinity chromatography in buffer F (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% DDM, 0.02% CHS). Finally, β2AR was exchanged to buffer G (20 mM HEPES, pH 7.5, 100mM NaCl, 0.01%LMNG, 0.002% CHS). All steps were performed at 4℃ with protease inhibitors benzamidine and leupeptin.

Sortase Ligation Reactions： All sortase reactions were conducted in buffer containing 20 mM HEPES, pH 7.5, 100 mM NaCl, 0.01% LMNG, 0.002% CHS, and 5 mM $CaCl_2$. Detergent-solubilized receptor (10 μM) was incubated with GGG-V2Rpp (GGGARGRpTPPpSLGPQDEpSCpTpTApSpSpSLAKDTSS) (50 μM) and 2 μM sortase A, Ligations were incubated overnight at 4℃, and unligated receptor (containing C-terminal 10xHis-tag) and sortase A were removed using Talon resin (Invitrogen). Size exclusion chromatography was utilized to specifically isolate monomeric ligated receptor.[5]

Expression and purification of Fab 30 proteins : The plasmid containing the gene for the 6× his tagged heavy and light chains of Fab30 cloned in the pETDuet-1 vector and was transformed into BL21(DE3) *E. coli* cells (Thermo, catalog number: EC0114). The cultured cells were then grown to an OD600 = 0.8 at 37 °C and induced with 500 μM IPTG at 18 °C for 16 h in the LB medium cultures. These cells were harvested by centrifugation and the cell pellets were lysed in buffer A (20 mM Tris-HCl, pH 8.0, 150 mM NaCl). The solution was poured into 50 ml centrifuge bottles and spin in SLA 1500 rotor for 30 min at 20,000×g. All remaining purification steps were carried out in cold room. The supernatant of the cell lysate was incubated with Ni-NTA beads by 2 h at 4 °C. The beads were packed in a column and washed with 40 CV of cold buffer B (20 mM Tris-HCl, pH 7.55, 150 mM NaCl), and then eluted with buffer C (20 mM Tris-HCl, pH 7.55, 150 mM NaCl, 250 mM imidazole). Dialyzed with 20 mM Tris-HCl, pH 7.55, 100 mM NaCl (buffer D) overnight and flash frozen with 10% glycerol.

Expression and purification of SeF incorporated βarr1: For the expression of SeF incorporated βarr1, plasmid pET22b/βarr1-6TAG mutant was co-transformed with pEVOL/SeFRS into BL21. *E. coli* cells were amplified at 37℃ in LB medium to OD600 of 1.0, and protein expression was induced with 0.3 mM IPTG, 0.02% L-arabinose and 0.5 mM SeF in the presence of 0.5 mM TCEP. After growing overnight at 25℃, the cells were harvested, and resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM imidazole). After sonication and centrifugation, the supernatant was purified by Co-NTA affinity chromatography and HiTrap™ heparin HP (GE healthcare).

Sequence of wt-βarr1:

MGDKGTRVFKKASPNGKLTVYLGKRDFVDHIDLVEPVDGVVLVDPEYLKERRVYVTLTCAFRYGREDLDVLGLTFRKDLFVANVQSFP
PAPEDKKPLTRLQERLIKKLGEHAYPFTFEIPPNLPCSVTLQPGPEDTGKACGVDYEVKAFCAENLEEKIHKRNSVRLVIRKVQYAPERP
GPQPTAETTRQFLMSDKPLHLEASLDKEIYYHGEPISVNVHVTNNTNKTVKKIKISVRQYADICLFNTAQYKCPVAMEEADDTVAPSSTF
CKVYTLTPFLANNREKRGLALDGKLKHEDTNLASSTLLREGANREILGIIVSYKVKVKLVVSRGGLLGDLASSDVAVELPFTLMHPKPKE
EPPHREVPEHETPVDTNLIELDTNDDDIVFEDFARQRLKGMKDDKEEEEDGTGSPRLNDRLEHHHHHH

Sequence of βarr1-T6SeF/L191-CCPGCC:

MGDKGSeFRVFKKASPNGKLTVYLGKRDFVDHIDLVEPVDGVVLVDPEYLKERRVYVTLTCAFRYGREDLDVLGLTFRKDLFVANVQS
FPPAPEDKKPLTRLQERLIKKLGEHAYPFTFEIPPNLPCSVTLQPGPEDTGKACGVDYEVKAFCAENLEEKIHKRNSVRLVIRKVQYAPE
RPGPQPTAETTRQFLCCPGCCMSDKPLHLEASLDKEIYYHGEPISVNVHVTNNTNKTVKKIKISVRQYADICLFNTAQYKCPVAMEEAD
DTVAPSSTFCKVYTLTPFLANNREKRGLALDGKLKHEDTNLASSTLLREGANREILGIIVSYKVKVKLVVSRGGLLGDLASSDVAVELPF
TLMHPKPKEEPPHREVPEHETPVDTNLIELDTNDDDIVFEDFARQRLKGMKDDKEEEEDGTGSPRLNDRLEHHHHHH

CaM labeling with Bodipy593 and Cy5: CaMF66SeF/M110C was diluted to 30 μM with buffer containing 50 mM Tris-HCl at pH 7.5, 150 mM NaCl, reduced by TCEP (60 μM, 2eq) for 5 min. Bodipy593 (150 μM, 5 eq) was added and the mixture was incubated for 30 min in dark. Then, Cy5-maleimide (300 μM, 10 eq) was added to the labeling mixture and incubated for another 1 h. The labeled CaM mutant was isolated from free dyes by Sephades G-25 column (NAP-5, GE). Labeling efficiency was estimated by measuring the absorbance at 280 nm, 565 nm and 649 nm. Labeled CaM was flash-frozen in liquid nitrogen and stored at -80℃. Ca^{2+} binding buffer contained 30 mM HEPES, 0.1 M KCl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.4. And Ca^{2+} free buffer contained 30 mM HEPES, 0.1 M KCl, 1 mM EDTA, pH 7.4.[6]

βarr1 mutant labeling with Bodipy593 and FlAsH-EDT$_2$: βarr1 mutant (30 μM) was incubated with TCEP (300 μM, 10 eq) at room temperature for 5 min. Bodipy593 (240 μM, 8 eq) and FlAsH (150 μM, 5 eq) were added to the mixture and incubated at room temperature for 1 h in dark. Free dyes were separated by Sephades G-25 column (NAP-5, GE). Labeling efficiency was established by measuring the absorbance at 280 nm, 565 nm, 510 nm. Labeled βarr1 was flash-frozen in liquid nitrogen and stored at -80℃.

Fluorescence Sepharose Exclusion Chromatography (FSEC) : βarr1 mutant (4 μM) was mixed with Fab30 (15 μM), V2Rpp or pp-β2V2R (5 eq) preincubated with supersaturated agonist (ISO) was added to the mixture. Fluorescence Sepharose Exclusion Chromatography experiments were performed on AKTA purifier equipped with Superdex 200 increase (10/300GL) column and FlAsH fluorescence detector. The sample volume was 200 μL. The sample was eluted with flow rate 0.35 mL/min in buffer (20 mM HEPES, 150 mM NaCl, 0.01% LMNG, 0.002% CHS, pH 7.5). The excitation and detection wavelength of the detector was 488 nm and 530 nm respectively.

Single-molecule fluorescence resonance energy transfer (smFRET) measurement: All smFRET experiments were performed at 25℃ with an oxygen scavenging system containing 3 mg/mL glucose, 100 μg/mL glucose oxidase (Sigma-Aldrich), 40 μg/mL catalase (Roche) and photostabilizing agents 1 mM cyclooctatetraene (COT, Sigma-Aldrich), 1 mM 4-nitro-benzylalcohol (NBA, Sigma-Aldrich), 1.5 mM 6-hydroxy-2, 5, 7, 8- tetramethyl-chromane-2-carboxylic acid (Trolox, Sigma-Aldrich). Single-molecule imaging was performed using a home-built objective-type total internal reflection fluorescence (TIRF) microscope, based on a Nikon Eclipse Ti-E with an EMCCD camera (Andor iXon Ultra 897), and solid state 488 nm, 532 nm, and 640 nm excitation lasers (Coherent Inc. OBIS Smart Lasers). Fluorescence emission from the probes was collected by the microscope and spectrally separated a Dual-View spectral splitter (Photometrics, Inc., Tucson, AZ). For Bodipy593/Cy5 and Cy3/Cy5 FRET pairs, a T635lpxr interference dichroic (Chroma), ET585/65m (Chroma, for Bodipy593 and Cy3) and ET700/75m (Chroma, for Cy5) bandpass filters were used. For FlAsH/Bodipy593 FRET pair, a T550lpxr interference dichroic (Chroma), ET525/50m (Chroma, for FlAsH) and ET585/65m (Chroma, for Bodipy593) band pass filters were used. Hardwares were controlled and smFRET movies were collected using Cell Vision software (Beijing Coolight Technology).
Collected movies were analyzed by a custom-made software program developed as an ImageJ plugin (http://rsb.info.nih.gov/ij). Fluorescence spots were fitted by a 2-D Gaussian function within a 9-pixel by 9-pixel area. The background subtracted total volume of the 2-D Gaussian peak was used as raw fluorescence intensity *I*. Single-molecule fluorescence traces displayed one of the following signatures were picked as the FRET traces to calculate FRET distributions. One is photobleaching of the donor leading the loss of both donor and acceptor signals. The other is the photobleaching of acceptor leading to the loss of FRET signal and a corresponding increase in donor signal. Usually, about 660 - 3000 FRET traces were used to calculate a FRET distribution.

smFRET measurement of βarr1 conformational changes: βarr1-K157SeF-Bodipy593/R418FlAsH (1 nm) was mixed with V2Rpp at different concentrations and incubated in binding buffer (50 mM Tris-HCl, pH 8.0, and 150 mM NaCl) at room temperature for 30 min. Then the mixture was incubated with biotin-anti his antibody (1:1) for 2 min and loaded on the slide, then washed with image buffer containing oxygen scavenging system. smFRET movies were collected.
Dual labeled βarr1 (SeF-Bodipy593/FlAsH and Cy3/Cy5) at 6 and 191 sites were incubated with biotin-anti his antibody (1:1) at room temperature for 2 min. Then the diluted sample (βarr1 1 nm) was loaded on a streptavidin covered slide. Image buffer containing pp-β_2V2R (1 μM), ISO (100 μM), Fab (20 nM), and oxygen scavenging system washed away the free βarr1. smFRET movies were collected.

Calculation of Förster radius (R_0): R_0 was calculated as follows, orientation factor κ^2 was assumed as 2/3, the refractive index was 1.33. Quantum yield: ϕ_{FlAsH} = 0.49, ϕ_{Cy3} = 0.31, $\phi_{SeF\text{-}Bodipy593}$ = 0.045. Mole extinction coefficient: $\varepsilon_{SeF\text{-}Bodipy593}$ = 123500 $M^{-1}cm^{-1}$, ε_{Cy5} = 250000 $M^{-1}cm^{-1}$. FlAsH/SeF-Bodipy593: R_0 = 60 Å; Cy3/Cy5: R_0 = 60 Å; SeF-Bodipy593/Cy5: R_0 = 43 Å

$$R_0^6 = 8.79 \times 10^{-5} (\kappa^2 n^{-4} Q_D J(\lambda)) \text{ (in } \text{Å}^6)$$

$$J(\lambda) = \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\,\lambda^4 d\lambda = \frac{\int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int_0^\infty F_D(\lambda) d\lambda}$$

Results and Discussion

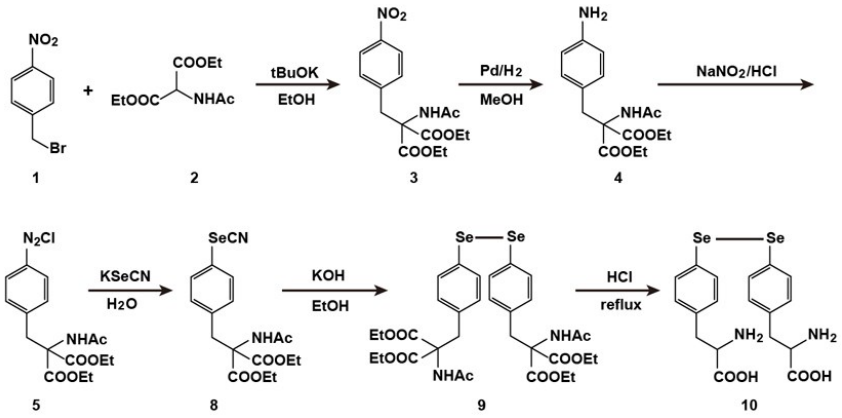


Fig. S1 Synthesis route of SeF.

Diethyl 2-acetamido-2-(4-nitrobenzyl) malonate 3: To a solution of diethyl 2- acetamidomalonate (7.815 g, 36 mmol) in EtOH (60 mL) was added potassium tert-butylate (4.399 g, 36 mmol), 1-(bromomethyl)-4-nitrobenzene (6.448 g, 30 mmol). The reaction mixture was reflux for 12 h, then cooled to room temperature. The yellow suspension was filtered, washed with EtOH. Compound **3** was obtained as yellow solid (8.451 g, 24 mmol, Y = 80%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.14 (d, *J* = 9.0 Hz, 2H), 7.19 (d, *J* = 9.0 Hz, 2H), 6.55 (s, 1H), 4.36 – 4.23 (m, 4H), 3.79 (s, 2H), 2.05 (s, 3H), 1.31 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.74, 167.43, 147.66, 143.50, 131.07, 123.85, 67.23, 63.40, 37.97, 23.41, 14.38. LCMS (ESI) calculated for C16H21N2O7 $[M+H]^+$: 353.1, found: 353.2.

Diethyl 2-acetamido-2-(4-aminobenzyl) malonate 4: Compound **3** (7.042 g, 20 mmol), 10% palladium on carbon (0.700 g), MeOH (100 mL) were added to a reaction tube which was capped in an autoclave, then the autoclave was purged and charged with H_2 at 4 atm. The reaction mixture was stirred at room temperature for 16 h. Then H_2 was carefully released and the reaction mixture was filtered through celite. After evaporation of the solvent under reduced pressure, the residue was purified by silica-gel column chromatography (hexane/ethyl acetate = 1:1) to afford the desired product **4** 6.247 g (Y = 97%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.79 (d, *J* = 8.5 Hz, 2H), 6.57 (d, *J* = 8.5 Hz, 2H), 6.54 (s, 1H), 4.31 – 4.20 (m, 4H), 3.62 (s, 2H), 3.53 (s, 2H), 2.02 (s, 3H), 1.29 (dd, *J* = 7.5, 7.0 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.29, 168.00, 145.80, 131.01, 125.14, 115.36, 67.72, 62.84, 37.41, 23.37, 14.35. LCMS (ESI) calculated for C16H22N2O5Na $[M+Na]^+$: 345.1, found: 345.1.

Diethyl 2-acetamido-2-(4-selenocyanatobenzyl) malonate 8: Compound **4** (1.0 g, 3.1 mmol) was stirred in 6 N HCl (5 mL). the reaction mixture was cooled to 0℃ , and 0.5 M aqueous $NaNO_2$ (256.7 mg, 3.72 mmol) was gradually added over 10 min. After stirring for a further 10 minutes, the pH of the solution is adjusted to 7.0 via the addition of Na_2CO_3. Then the reaction mixture was cooled to 0℃, and was added aqueous KSeCN (490.0 mg, 3.41 mmol) dropwise. After stirring at room temperature for 1 h, the resulting mixture was extracted with CH_2Cl_2 (150 mL). The combined organic phases were washed with H_2O and brine, dried over Na_2SO_4. The solution was evaporated and purified by silica-gel chromatography (hexane/ethyl acetate = 2:1) to obtained the product **8** 766 mg (Y = 60%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 (d, *J* = 8.0 Hz, 2H), 7.06 (d, *J* = 8.0 Hz, 2H), 6.55 (s, 1H), 4.34 – 4.21 (m, 4H), 3.68 (s, 1H), 2.04 (s, 2H), 1.30 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.62, 167.55, 137.98, 133.06, 132.18, 120.90, 101.58, 77.61, 77.36, 77.11, 67.36, 63.24, 37.72, 23.41, 14.37. LCMS (ESI) calculated for C17H21N2O5Se $[M+H]^+$: 413.1, found: 413.2.

3,3'-(diselanediylbis(4,1-phenylene))bis(2-aminopropanoic acid) 10: KOH (521.7 mg, 9.3 mmol) was dissolved in 10 mL EtOH, diethyl 2-acetamido-2-(4-seleno-cyanatobenzyl) malonate **8** (766 mg, 1.86 mmol) was added under an atmosphere of N_2, the reaction mixture was stirred at room temperature for 1 h and H_2O (10 mL) was added to the mixture. Compound **9** was afforded by filtrating the precipitate. Compound **9** was dissolved in concentrated HCl (10 mL) and refluxed for 16 h, After neutralization the resulted mixture with NaOH, the precipitate was formed and filtrated to afford the final product **10** 487.9 mg (Y = 71%). ^{1}H NMR (500 MHz, D_2O) δ 7.49 (d, *J* = 8.0 Hz, 2H), 7.08 (d, *J* = 7.5 Hz, 2H), 3.41 (dd, *J* = 8.3, 5.0 Hz, 1H), 2.98 (dd, *J* = 13.5, 4.8 Hz, 1H), 2.67 (dd, *J* = 13.4, 8.5 Hz, 1H). ^{13}C NMR (126 MHz, D_2O) δ 181.89, 138.43, 132.48, 130.27, 128.79, 57.40, 40.68. LCMS (ESI) calculated for C18H21N2O4Se2 $[M+H]^+$: 488.9, found: 488.9.

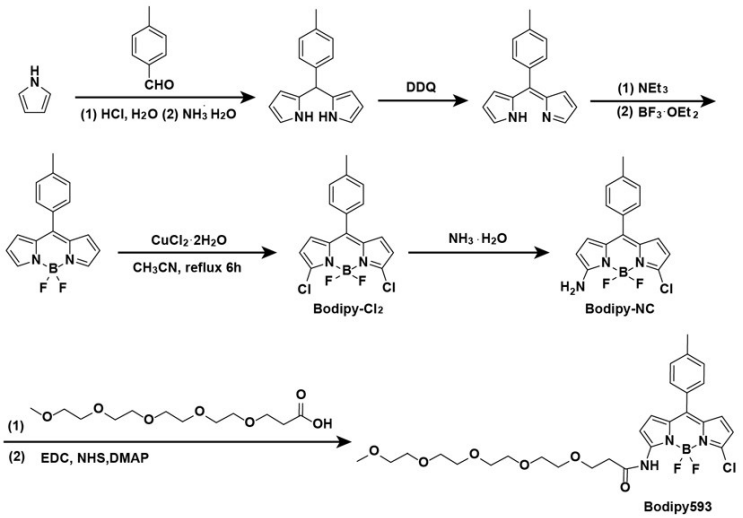


Fig. S2 Synthesis route of Bodipy593.

Bodipy-Cl_2 was synthesis as previous report.[7]

Bodipy-NC: Bodipy-Cl_2 (350 mg, 1.0 mmol) was dissolved in dry acetonitrile (10 mL) under nitrogen. 25% ammonia (763 μL, 5.0 mmol) was added dropwise with stirring. After stirring at room temperature for 3 h, the reaction was quenched with 200 mL water and precipitation was filtered, then dried by vacuum. The product is purified through column chromatography over silica (dichloromethane/petroleum ether = 5/1 as eluent) to give compound **3** (275 mg, 0.83 mmol, 83%) as a red solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35 (d , J = 8.0 Hz, 2H), 7.26 (d, J = 8.0 Hz, 2H), 6.89 (d, J = 4.8 Hz,1H), 6.39 (d, J = 4.0 Hz,1H), 6.19 (d, J = 4.0 Hz, 1H), 6.10 (d, 1H, J = 4.8 Hz), 5.84 (br, 2H), 2.43 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.3, 139.7, 135.9, 133.6, 133.2, 132.1, 130.9, 130.4, 129.1, 121.1, 113.3, 113.0, 21.5; HRMS(ESI): calculated for $[M + H]^+$: 332.0935, found: 332.0938.

Bodipy593: Bodipy-NC (66.2 mg, 0.2 mmol) was dissolved in dry acetonitrile (30 mL) under nitrogen. 2,5,8,11,14-pentaoxaheptadecan-17-oic acid (56.0 mg, 0.2 mmol), DCC (61.8 mg, 0.3 mmol), NHS (17.3 mg 0.15 mmol), DMAP (18.3 mg, 0.15mmol) and Na_2CO_3 (21 mg, 0.2 mmol) was added respectively with stirring. After stirring at room temperature for 48 h, the reaction was quenched with saturated salt water and extracted with dichloromethane. The organic layer was dried over Na_2SO_4, concentrated and purified through column chromatography over silica (DCM/CH_3OH= 95/5 as eluent) to give Bodipy593 (24.9 mg, 0.042 mmol, 21%). LRMS: calculated for $C_{26}H_{31}BClF_2N_3NaO_5$ $[M + Na]^+$: 616.22, found: 616.33.

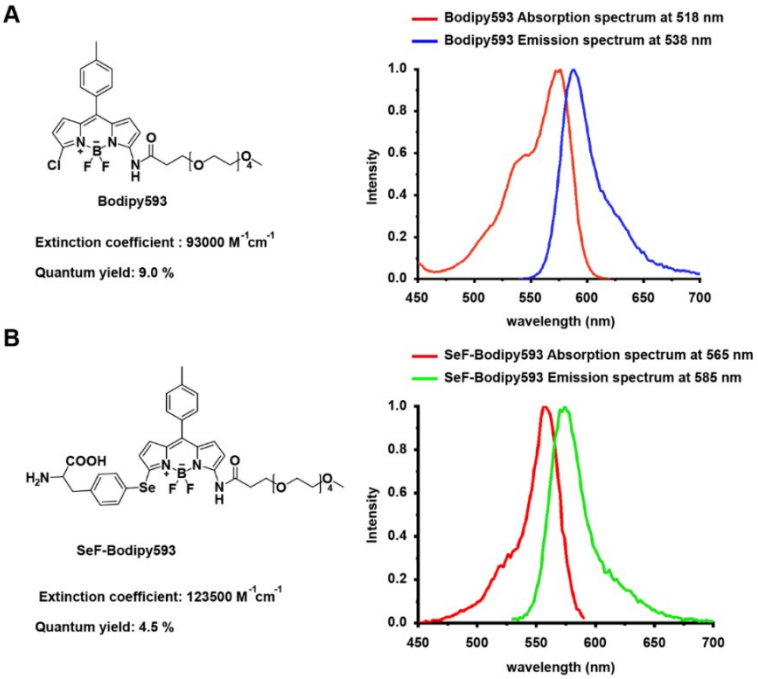


Fig. S3 Chemical structure and optical characterization of Bodipy593 (A) and SeF-Bodipy593 (B). Measurement of extinction coefficient**:** Bodipy593 (4.0 mg) was added to CH_3CN (1 mL) and rotated to dissolve absolutely. The stock solution (2 μL) was diluted by 10% CH_3CN in H_2O (998 μL) in dark, and subjected to uv-vis spectrum measurement to afford the max absorbance 1.3562 at 518 nm, coefficient extinction was 93000 $M^{-1}cm^{-1}$. At the same concentration, the max absorbance of SeF-Bodipy593 is 1.7977, and the coefficient extinction is 123500 $M^{-1}cm^{-1}$. Quantum yield was measured using Cy3 (quantum yield: 0.1; excitation max: 548 nm; emission max: 563 nm, lumiprobe Co. number 11380) as standard.

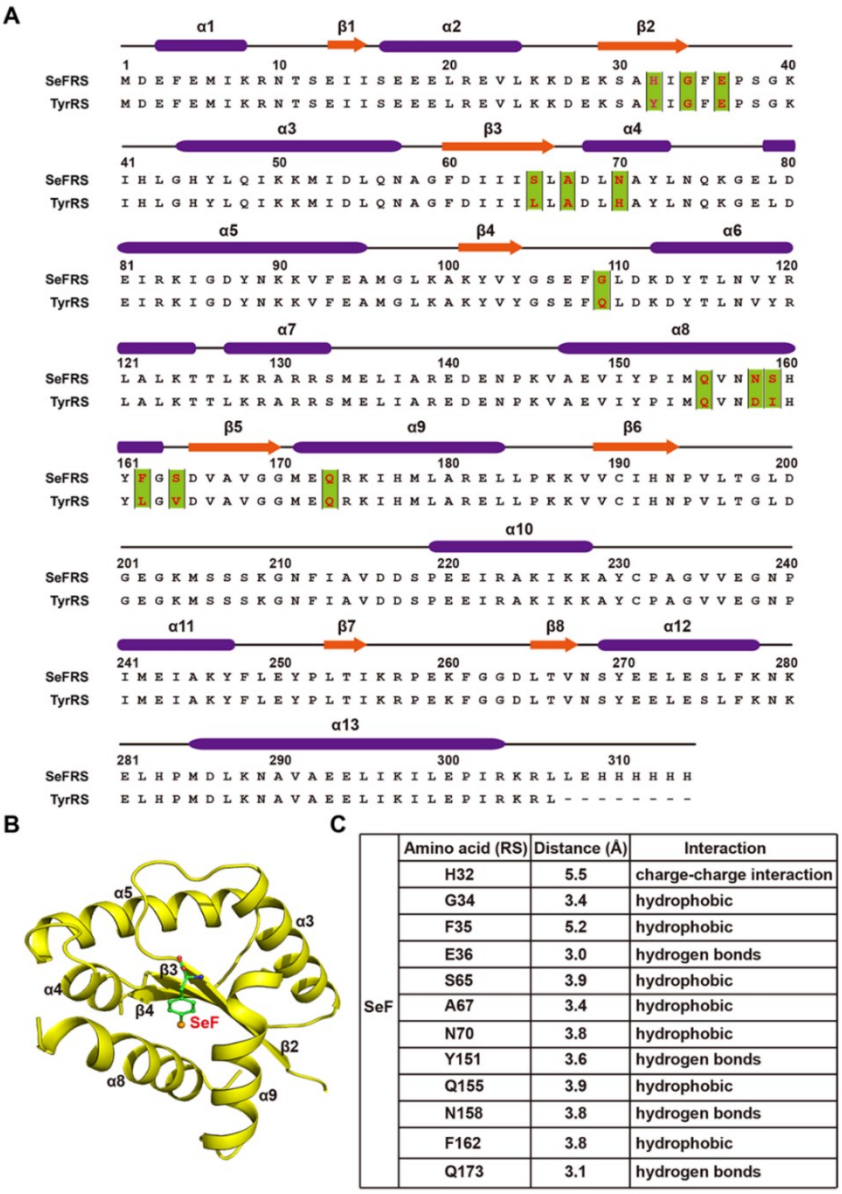


Fig. S4 Sequence alignment between SeFRS and TyrRS and the interactions for SeF in SeFRS. (A) The sequence alignment among SeFRS and TyrRS. The secondary structure β-strands were marked in orange arrows and α-helices were indicated in purple boxes. The labeled resides indicate the momentous interactions for SeF in SeFRS. (B) Structure of SeF and SeFRS complex. The SeFRS is shown in yellow cartoon representation. SeF is shown as sticks in green. (C) The interactions including hydrophobics, hydrogen bonds, charge-charge interaction are shown between SeF and SeFRS.

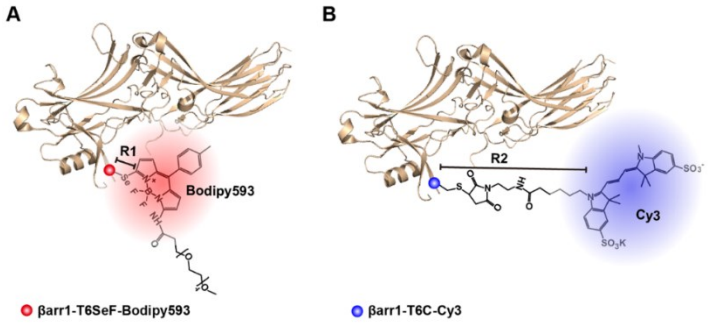


Fig. S5 Linker comparation between Se-Click mediated Se-Bodipy593 labeling (A) and popular cysteine-maleimide mediated Cy3 labeling (B). Bond length of C-Se is 1.93 Å, and linker of SeF-Bodipy593 labeling is 3.4 Å.

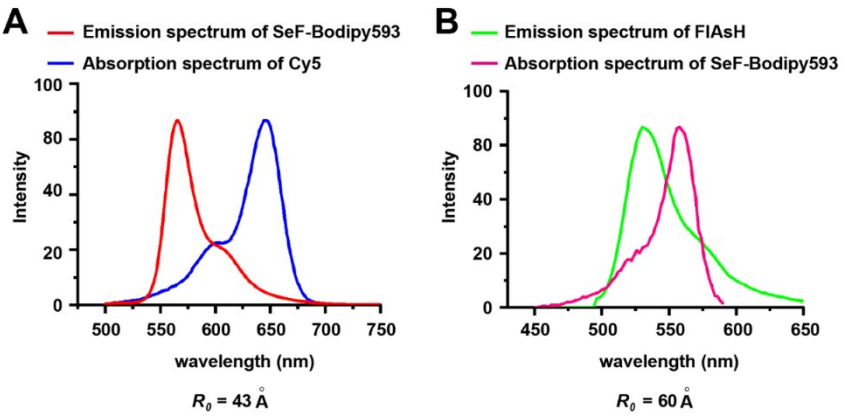


Fig. S6 Normalized donor emission spectra and acceptor absorption spectra for SeF-Bodipy593/Cy5 (SeF-Bodipy593: red, Cy5 blue) (A) and FlAsH/SeF-Bodipy593 (FlAsH: Green; SeF-Bodipy593: purple) (B) FRET pairs. The spectral overlap integral was calculated and used to identify the Förster Radius (R_0) for each pair.

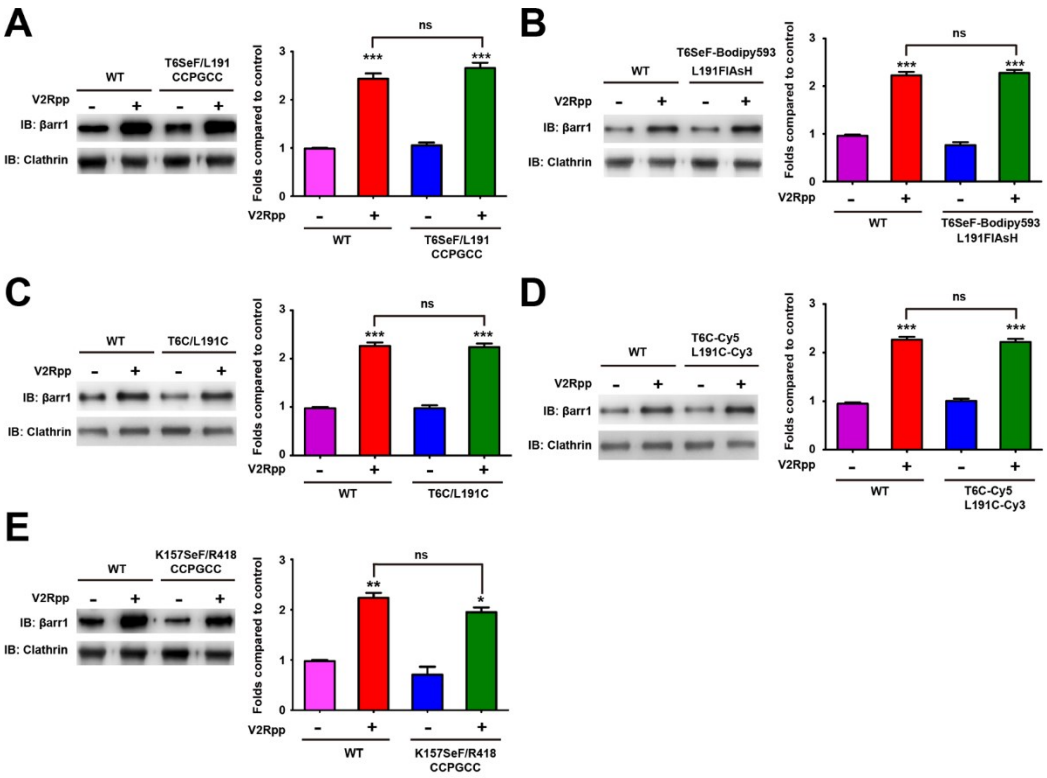


Fig. S7 Effects of βarr1 mutations on V2Rpp promoted βarr1/clatharin complex formation. Western blot assay was performed as previous report.[1] The western blot signal of βarr1 bound to clathrin were quantified. Data are shown as mean ± s. e. m for three independent experiments.

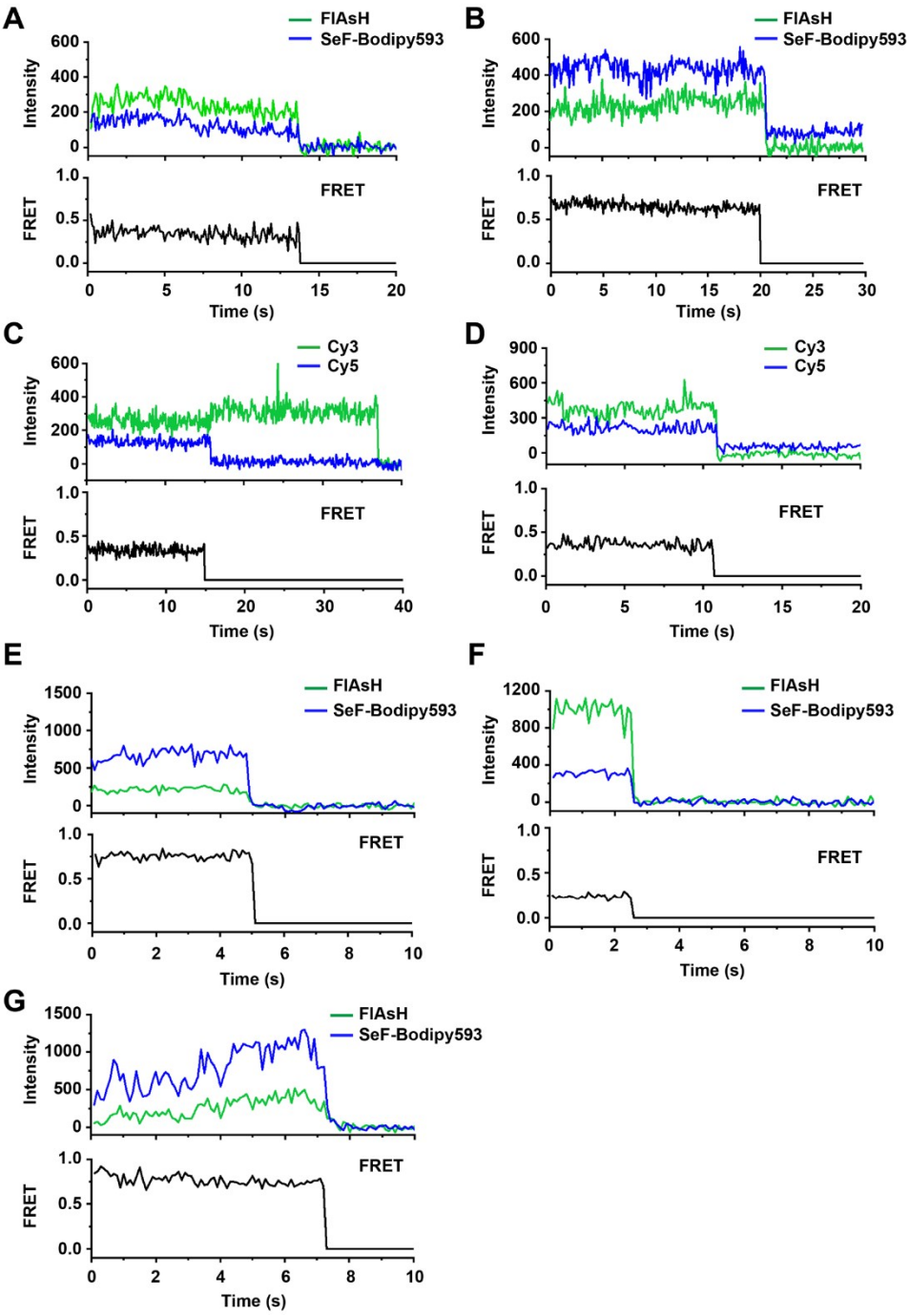


Fig. S8. Sample fluorescence and FRET time traces for β-arrestin-1 before and after activation. βarr1K157SeF-Bodipy593/R418-FlAsH before (A) and after (B) activation by V2Rpp; βarr1-T6C/L191C-(Cy3/Cy5) before (C) and after (D) activation by pp-β2V2R; βarr1-T6SeF-Bodpy593/L191-FlAsH before (E) and after (F, G) activation by pp-β2V2R.

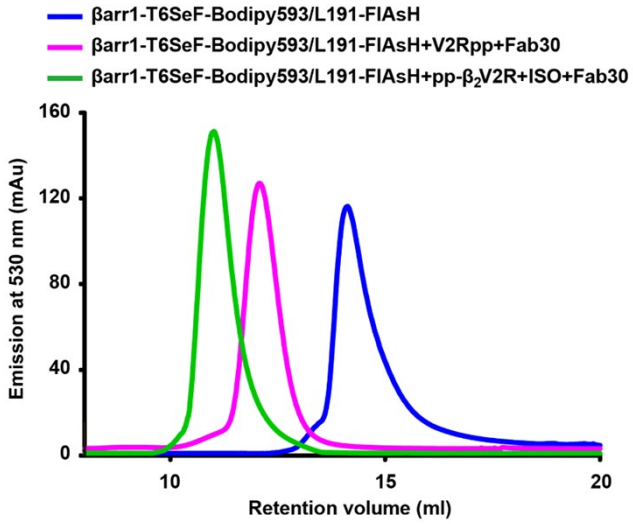


Fig. S9 Fluorescence Sepharose Exclusion Chromatography (FSEC) assay. This experiment confirmed the formation of (βarr1-T6SeF-Bodipy593/L191- FlAsH)-V2Rpp-Fab30 complex and (βarr1-T6SeF- Bodipy593/L191-FlAsH)-pp-β_2V2R-Fab30 complex.

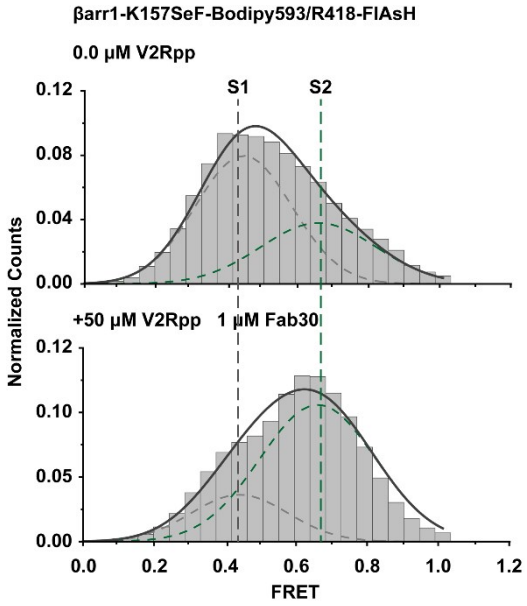


Fig. S10 smFRET distributions of βarr1-K157SeF-Bodipy593/R418-FlAsH. before and after V2Rpp activation in the presence of Fab30. Dashed lines highlight the mean FRET values.

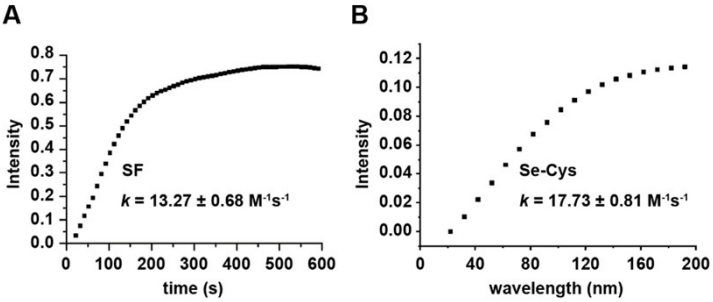


Fig. S11 Kinetic measurement of the reaction rate between SF(2-amino-3-(4-mercaptophenyl) propanoic acid) and Bodipy593 (A); Se-Cys (2-amino-3-hydroselenopropanoic acid) and Bodipy593 (B). Data are shown as mean ± s. e. m. Curves are representative of three independent experiments.

Table S1. Enzymatic reaction applied on protein modification.[8,9]

Enzymatic reaction	Tag size (kDa)	*Kcat/Km*	linker
SNAP-tag	20	10^4	7
CLIP-tag	17	10^3	7
HAlo-tag	13	10^6	12
TMO-tag	>20	10^3	—
Lipoic acid ligase	>10	10^2	6
Sortase	LPXTG motif	—	pentaglycine

Table S2. Bioorthogonal reactions enabled by unnatural amino acid incorporation.

Bioorthogonal reaction	Kinetics ($M^{-1}s^{-1}$)	Unnatural amino acid (UAA)	Number of atoms changed	Linker length	New stereo isomer	Catalyst requirement
Se-click reaction	26.3 ± 0.4	Selenophe （SeF)	1	1	N	N
Inverse electron demand Diels–Alder reactions (IEDDA)[10]	1 - 10^6	norbornene[11]	>7	> 2	Y	N
		Trans-cyclooctene (TCO)[12]	>11	> 3	Y	N
		cyclopropene[13]	8	> 4	Y	N
		tetrazine[14]	>8	> 1	Y	N
		spiro[2.3]hex-1-ene(Sph)[15]	>10	> 6	Y	N
Diels–Alder reactions	1-10^4	BCN[16]	13	>7	Y	N
		SCO[12]	11	>3	Y	N
Azide-alkyne Cu(I)	10 - 100	azide[17]	3	> 3	N	Cu(I)
		alkyne[18]	2	> 3	N	Cu(I)
SPAAC	10^{-2} - 1	cyclooctyne[19]	11	> 5	N	N
Photoclick cycloadditions	10 - 60	tetrazole[20]	5	> 3	Y	light
Staudinger reaction	10^{-4}	azide[21]	3	> 2	N	N
Ketone/alkoxyamine or hydrazine condensation[22]	10^{-4} - 10^{-3}	ketone[17]	3	> 3	N	N

Table S3. Data collection and refinement statistics of the SeFRS (PDB: 7C5C).

Data collection and processing			
Cell dimensions			
a, b, c (Å)	**52.61**	**38.78**	**82.31**
α, β, γ (°)	**90**	**91**	**90**
Space group	**P*121***		
Resolution range (Å)	**44.73-1.72 (1.78—1.72)**		
Unique reflections	**35043 (2851)**		
Multiplicity	**6.3 (4.2)**		
I/σ	**28.94 (2.89)**		
Rmerge (%)[a]	**3.1 (33.4)**		
Completeness (%)	**97.83 (80.22)**		
Refinement			
Resolution (Å)	**44.73-1.72 (1.78—1.72)**		
Rwork[b]/ Rfree[c]	**0.19（0.30）/ 0.22（0.37）**		
Protein atoms	**2501**		
Solvent atoms	**203**		
Mean B factor (Å^2)	**43.87**		
R.M.S. deviations			
Bond lengths (Å)	**0.005**		
Bond angles (°)	**0.77**		
Ramachandran plot[d]			
Most favored (%)	**99.03**		
Additional allowed (%)	**0.97**		
outliers (%)	**0.00**		

Table S4. Photophysical properties comparison between SeF-Bodipy593 and Cy3. SeF-Bodipy593 measurement was performed on CaM66SeF-Bodipy593, and Cy3 was on ribosomal L11 protein.[23] Total dwell time before photobleaching, total photon collected before photobleaching and average SNR of individual fluorophores at 100 ms per frame. Standard error of mean (SEM) is shown.

Dye	Lifetime (s)	Total photon	S/N ratio	Excitation laser
Bodipy593	3.6 ± 0.1 s	24204 ± 333	3.89 ± 0.07	0.2 μW μm^{-2} 532 nm
Cy3	3.3 ± 0.1 s	28513 ± 774	6.28 ± 0.04	0.2 μW μm^{-2} 532 nm

Table S5. Calculations formulas for polarization degree: Polarization degree measurement was performed on F-7000 FL Spectrophotometer, using single fluorophore labeled protein in tris buffer (50 mM tris, 150 mM NaCl, pH = 7.4). SeF-Bodipy593 and FlAsH were excited at 555 nm and 508 nm, Cy3 and Cy5 were excited at 550 nm and 649 nm respectively. Each data was collected at apex. Calculation was as follows:

$$G = I\perp / I\parallel \qquad P(\lambda) = \frac{I\parallel(\lambda) - G*I\perp(\lambda)}{I\parallel(\lambda) + G*I\perp(\lambda)} \qquad R(\lambda) = \frac{I\parallel(\lambda) - G*I\perp(\lambda)}{I\parallel(\lambda) + 2G*I\perp(\lambda)}$$

	Excitation	Emission
$I\perp$	90°	0°
$I\parallel$	90°	90°
$I\parallel(\lambda)$	0°	0°
$I\perp(\lambda)$	0°	90°

Table S6. Measurement of polarization degree. The G, P, R data are the mean of 3 independent Experiments, and all the error bars are less than 0.01. All seven cysteines were mutated to alanine and named βarr1 no C.

Sample	wavelength (Ex/Em, nm)	G	P	R
βarr1-T6SeF-Bodipy593	555/580	1.67	0.36	0.27
βarr1 no C-T6C-Cy3	560/573	1.63	0.38	0.29
βarr1 no C-T6C-Cy5	649/684	1.89	0.31	0.23
βarr1-L191-FlAsH	508/533	1.61	0.36	0.27
βarr1 no C-L191C-Cy3	550/571	1.67	0.40	0.31
βarr1 no C-L191C-Cy5	650/676	1.89	0.34	0.26
CaM-F65SeF-Bodipy593	555/572	1.65	0.29	0.22
CaM-F65C-Cy3	550/570	1.66	0.36	0.27
CaM-F65C-Cy5	650/680	1.87	0.30	0.23

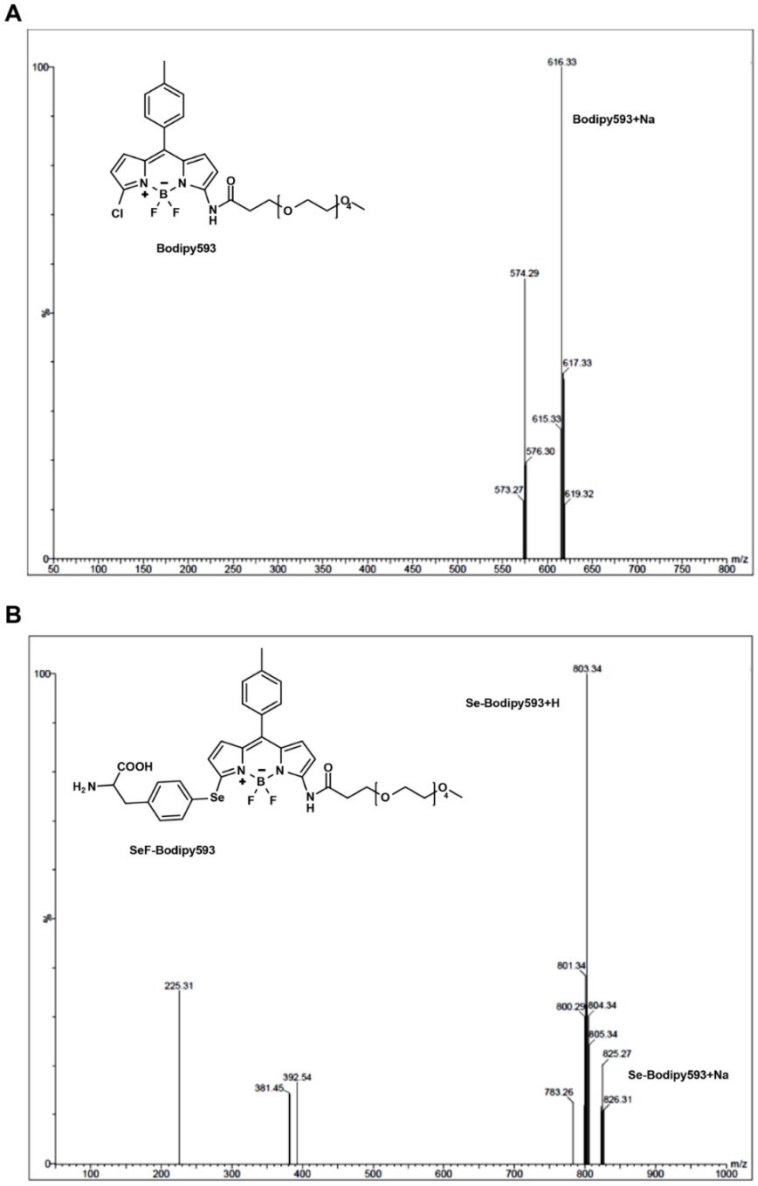
Table S7. Compared the Cα distance of βarr1-T6-L191 between inactive and active states.[24-28]

PDB	Distance between T6 and L191 (Å)	Inactive or active	Bound GPCR phosphopeptide	Bound GPCR	Resolution (Å)	Reference
1G4M	Chain A: 63.6 Chain B: 63.3	Inactive	-/-	-/-	1.9	Han et al., Structure. 2001[7c]
2WTR	Chain A: 64.0 Chain B: 63.5	Inactive	-/-	-/-	2.9	-/-
6KL7	Chain A: 63.8 Chain B: 64.0	Inactive	-/-	-/-	2.8	Kang et al., Structure.2020[7d]
4JQI	70.6	Active	V2Rpp	-/-	2.6	Shukla., Nat. 2013[2]
6NI2	69.9	Active	-/-	β2V2R	4.0	Nguyen et al., Nat. Struct. Mol. Biol. 2019[7b]
6U1N	72.4	Active	-/-	M2R	4.0	Staus et al., Nat. 2020[7a]

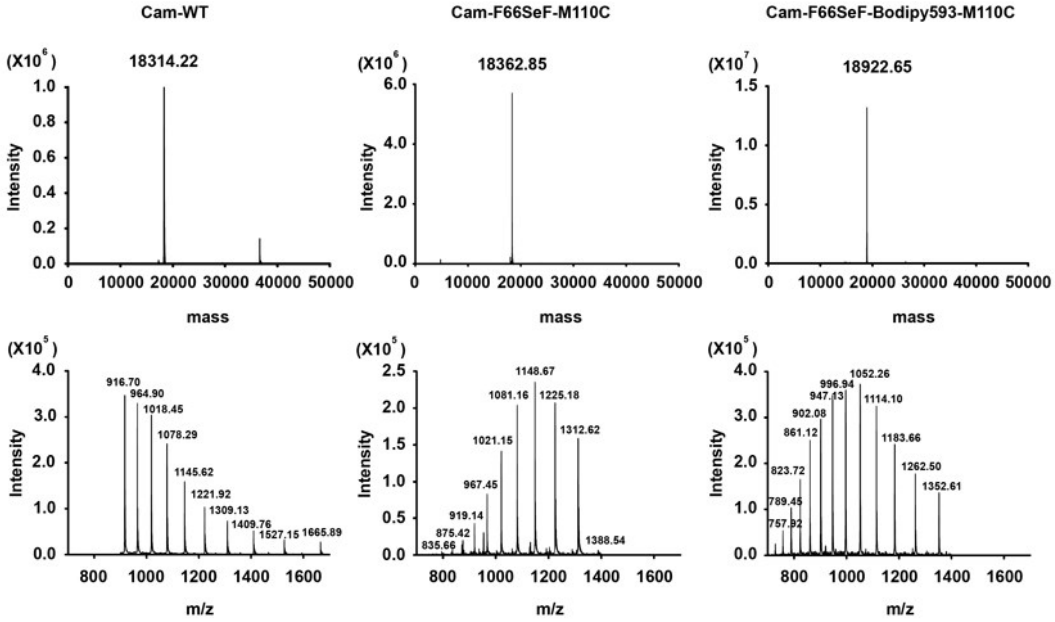
Table S8. The accessible volume (AV) simulation to compute donor-acceptor distances: The model for βarr1 (PDB: 6NI2) was used to simulate AVs of the dyes via a freely available software.[29] Labeling sites were βarr1-T6SeF-Bodpy593/L191-FlAsH and βarr1-T6C-Cy5/L191C-Cy3. The atomic number of T6 in βarr1 were (125.541, 95.705, 93.229), and the atomic number of L191 in βarr1 were (78.304, 89.200, 42.093). In the software,[30] the connecting linker is modeled as a flexible cylinder with linker length and linker width. The fluorophore is approximated by an ellipsoid with defined radii R1, R2, and R3. Following the recommended value given by the software, the following parameters were used for our AV simulations.

	Linker length (Å)	Linker width (Å)	R1(Å)	R2(Å)	R3(Å)
SeF-Bodipy593	7	4.5	4.5	6.4	0.9
FlAsH	7	4.5	4.5	6.4	0.9
Cy3	21	4.5	10.0	4.7	1.5
Cy5	21	4.5	11.0	4.7	1.5

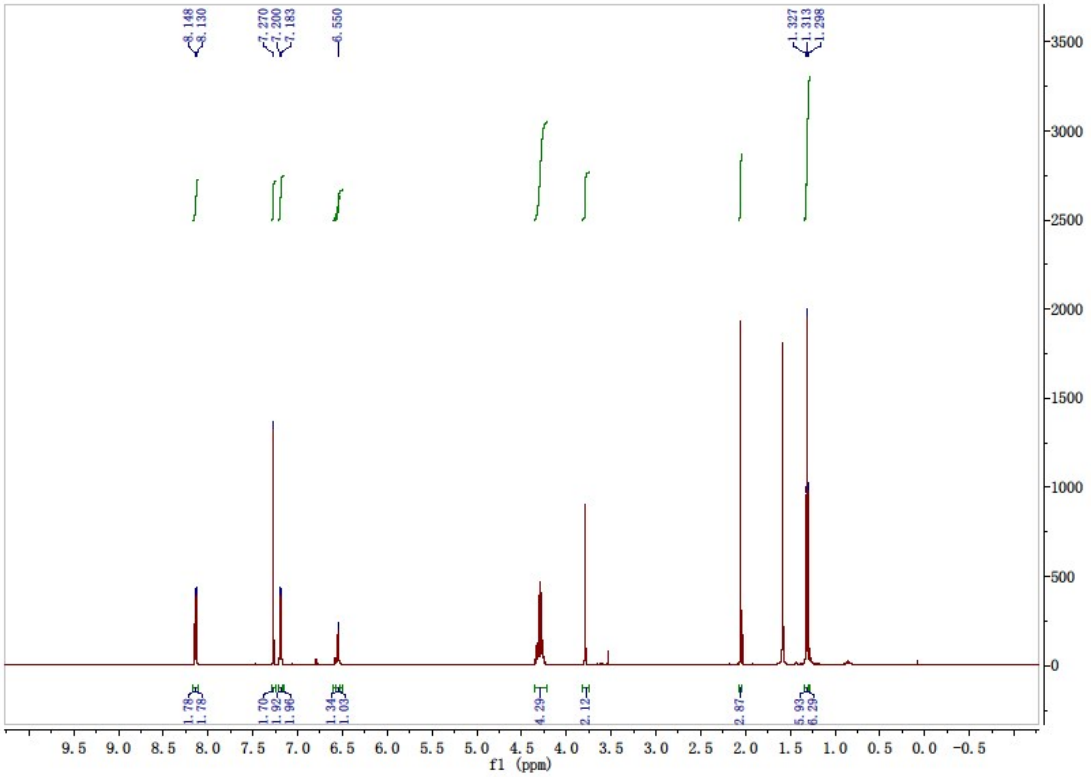
MS analysis of Bodipy593 (A) and SeF-Bodipy593 (B)



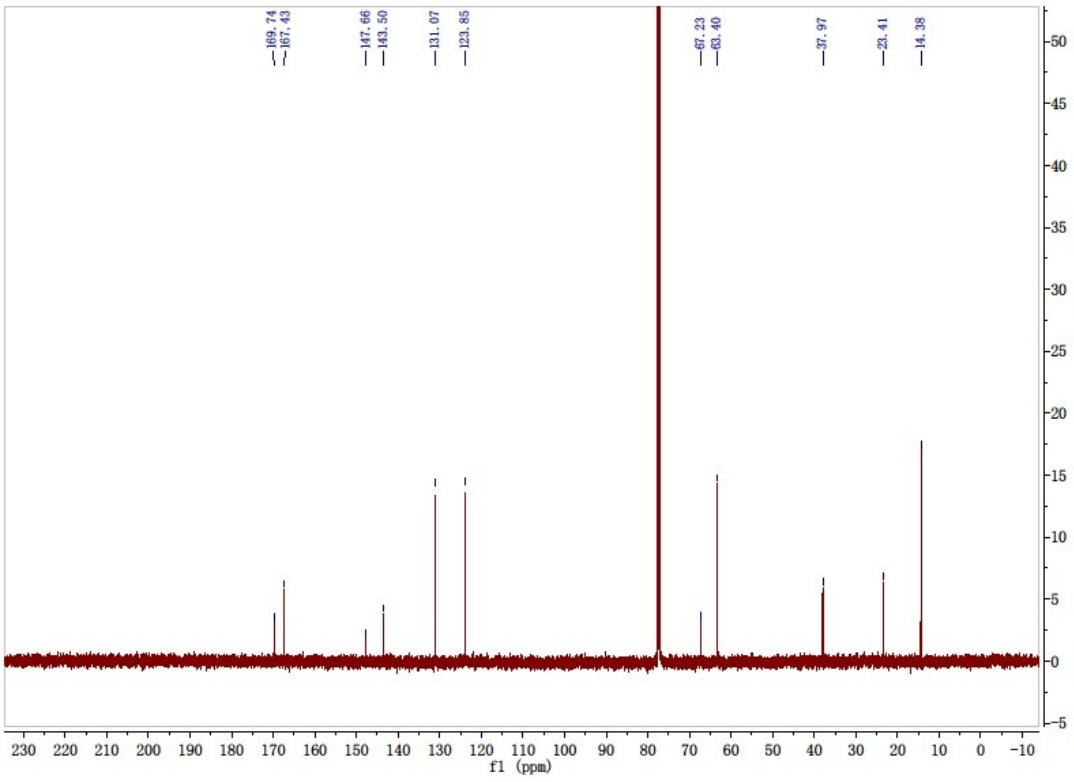
MS analysis of CaM mutant



Diethyl 2-acetamido-2-(4-nitrobenzyl)malonate 3 ^{1}HNMR



Diethyl 2-acetamido-2-(4-nitrobenzyl)malonate 3 ^{13}CNMR



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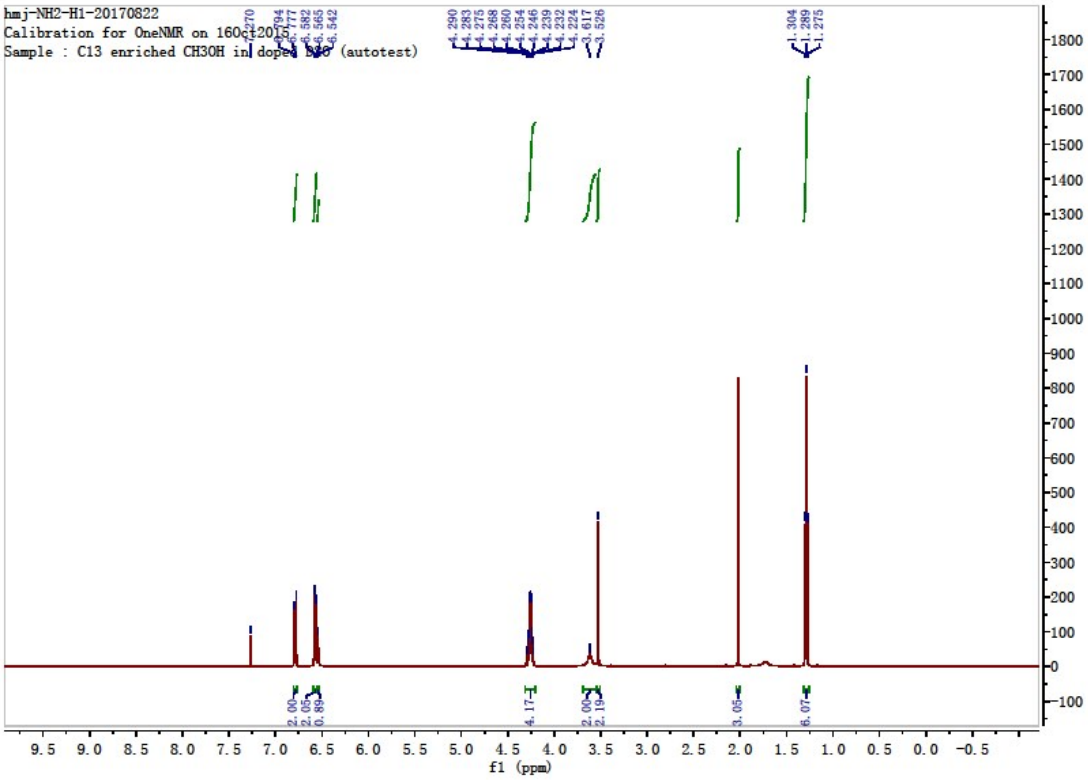
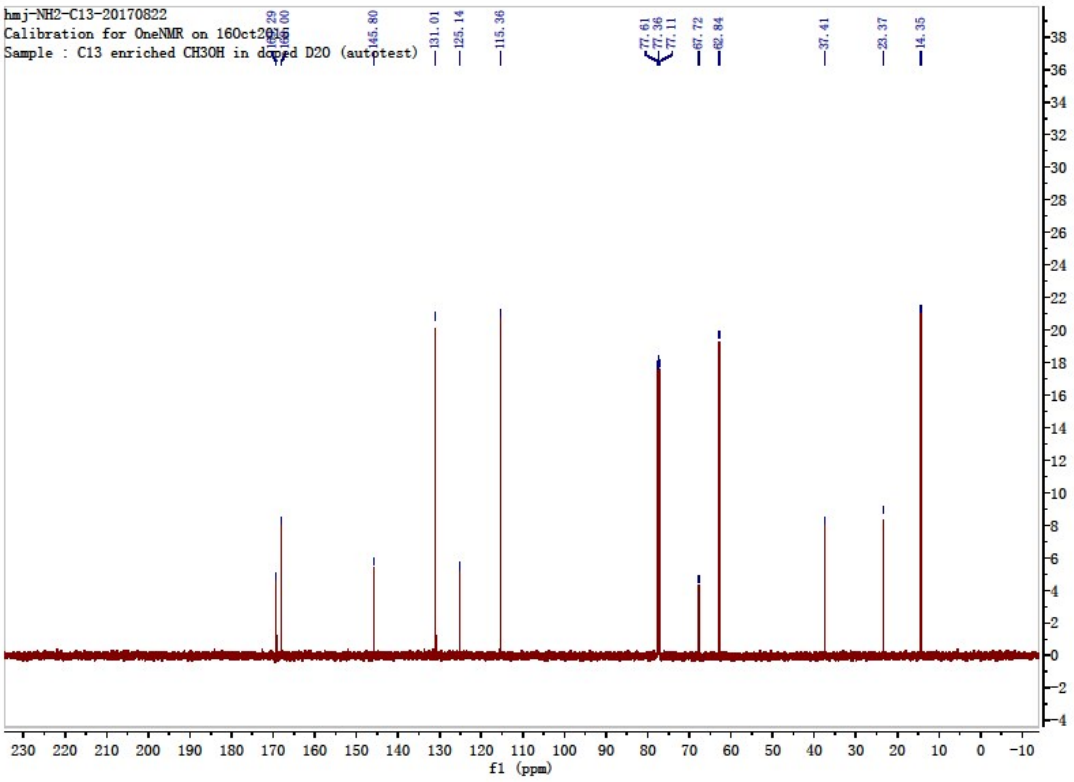
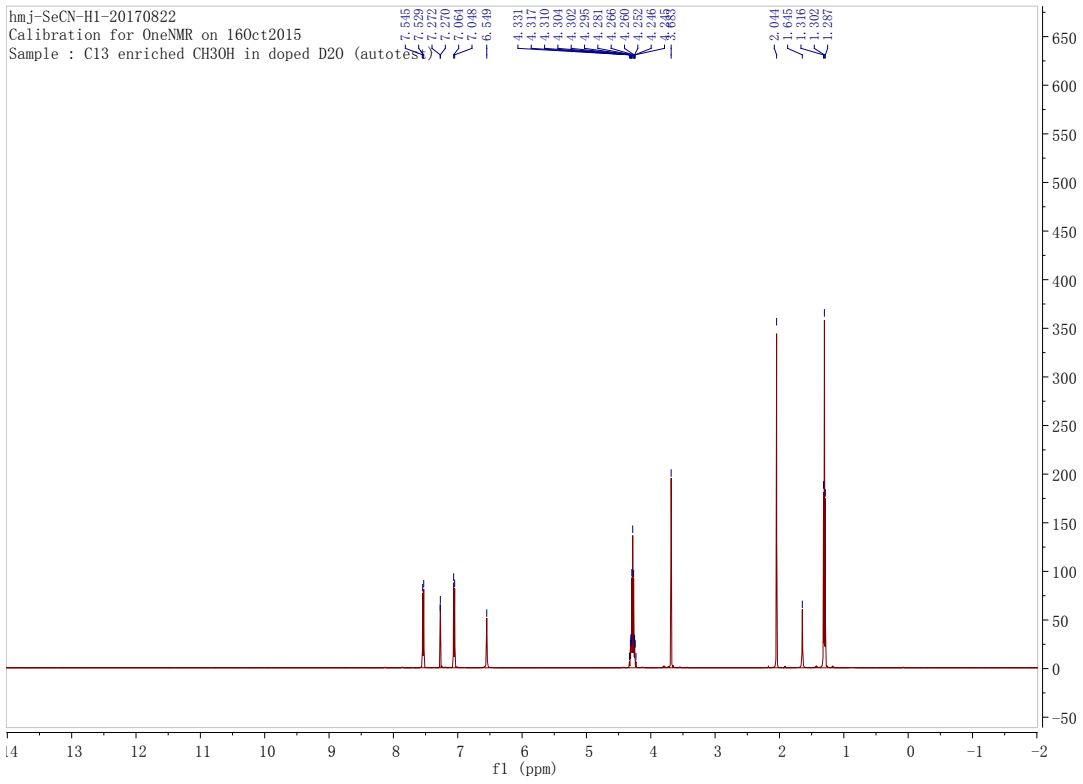


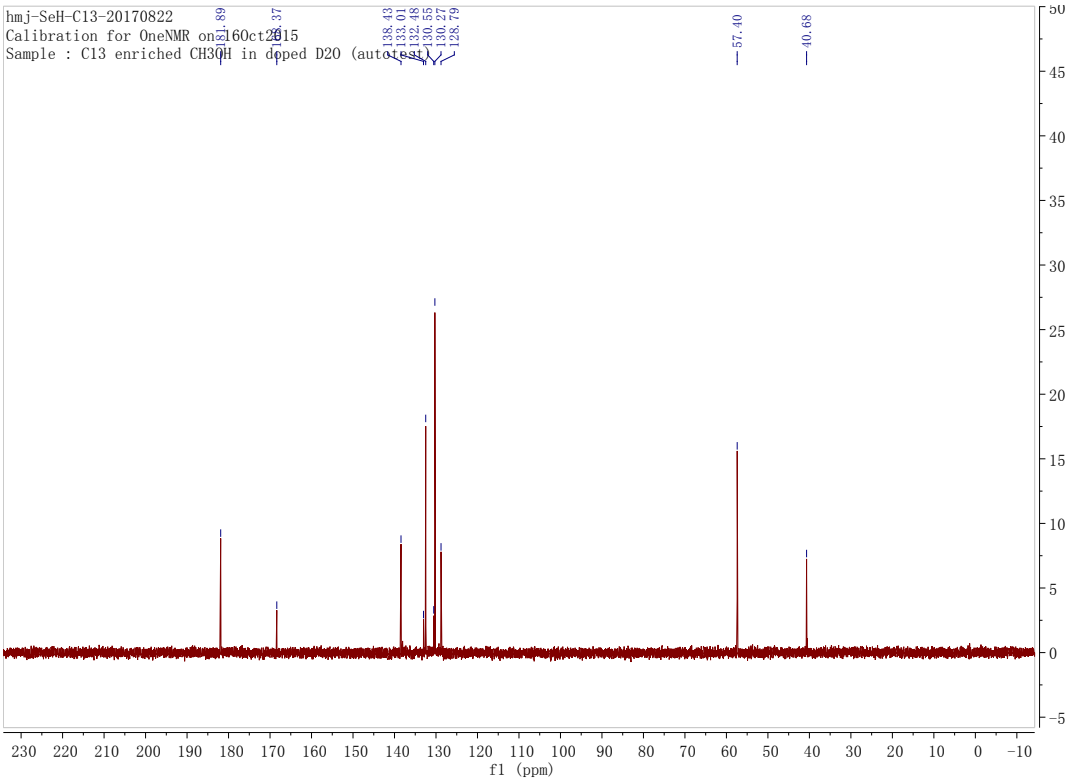
Diethyl 2-acetamido-2-(4-aminobenzyl) malonate 4 ^{13}CNMR



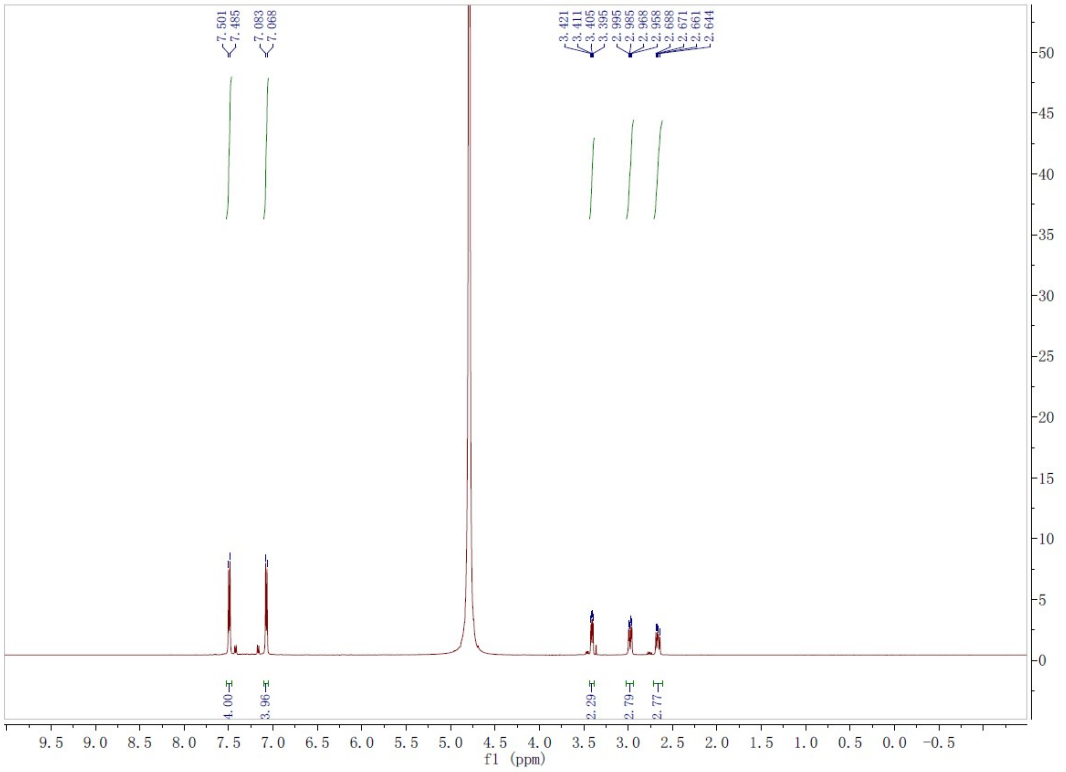
Diethyl 2-acetamido-2-(4-selenocyanatobenzyl) malonate 8 ^{1}HNMR



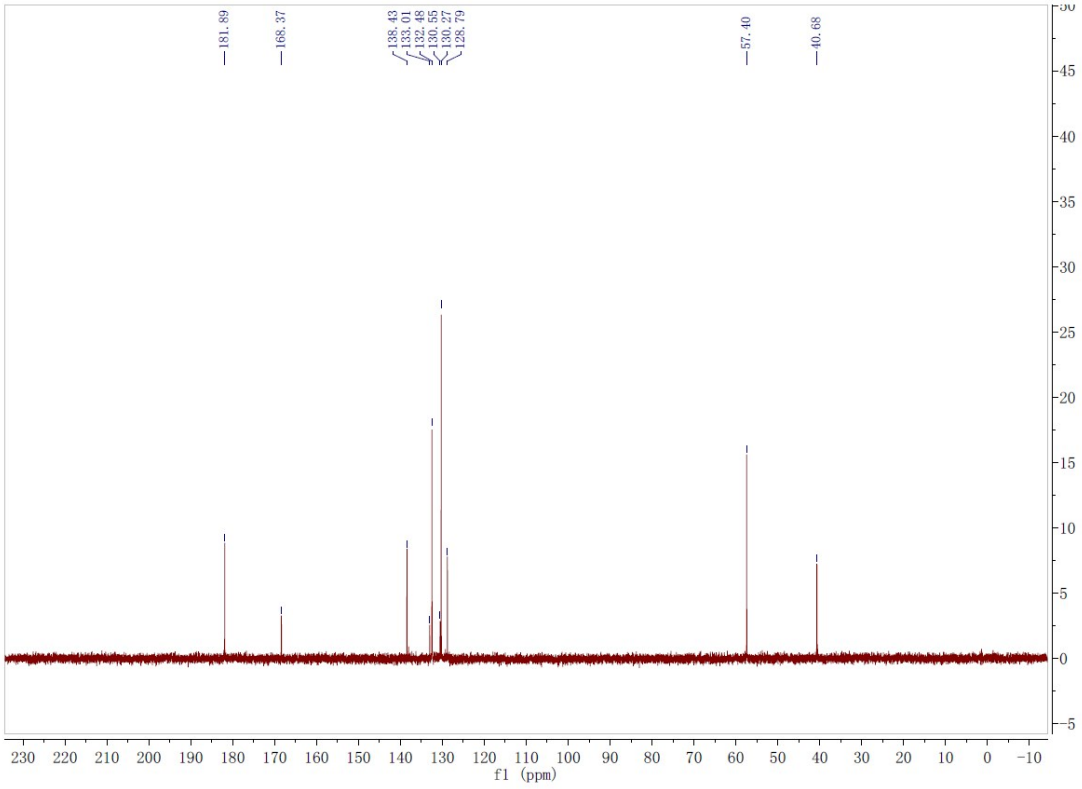
Diethyl 2-acetamido-2-(4-selenocyanatobenzyl) malonate 8 ^{13}CNMR



3,3'-(diselanediylbis(4,1-phenylene))bis(2-aminopropanoic acid) 10 ^{1}HNMR



3,3'-(diselanediylbis(4,1-phenylene))bis(2-aminopropanoic acid) 10 ^{13}CNMR



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