Supporting Information

Photoswitchable Serotonins for Optical Control of the 5HT2A Receptor

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Reagents and Instrumentation

All reagents and solvents were purchased from commercial sources (Acros Organics, Alfa Aesar, Cayman, Combi-Blocks, Oakwood, Sigma Aldrich, TCI, TRC, etc.) and were used without further purification. Monodisperse PEG-linkers were purchased from PurePEG. Solvents were obtained from Fisher Scientific. Reactions were monitored by thin layer chromatography (TLC) on glass plates precoated with silica gel (0.25 mm, 60 Å pore size, Merck). The plates were visualized by exposure to UV light (254 nm). Flash silica gel chromatography was performed on a CombiFlash EZ Prep™ using silica gel (SiO_2, particle size 40-63 µm) purchased from SiliCycle. NMR spectra were measured on a Bruker AV-III HD 400 MHz (equipped with a CryoProbe™) (operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C) or on a Bruker AVIII-600 High Performance Digital NMR Spectrometer with a CPTCI-cryoprobe head (600 MHz for ^{1}H and 150 MHz for ^{13}C). Multiplicities in the following experimental procedures are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. ^{1}H chemical shifts are expressed in parts per million (ppm, δ scale). The residual protium in the deuterated solvent was used as internal reference (MeOD: δ = 7.26 or $CDCl_3$: δ = 77.16). ^{13}C chemical shifts are expressed in ppm (δ scale) and are referenced to the carbon resonance of the NMR solvent (MeOD: δ = 49.00 or $CDCl_3$: δ = 77.16). Structural analysis was conducted with 1H- and 13C-NMR spectra with the aid of additional 2D spectra (COSY, HMBC, HSQC). Spectra analysis was conducted with the software MestReNova. NOTE: Due to the *trans*/*cis* isomerization of some compounds containing an azobenzene functionality, more signals were observed in the ^{1}H and ^{13}C spectra than would be expected for the pure *trans*-isomer. Only signals for the major *trans*-isomer are reported. High-Resolution Mass Spectra (HRMS) were recorded on an Agilent 6224 Accurate-Mass TOF/LC/MS using an electrospray ionization source (ESI). FTIR were recorded on a ThermoScientific Nicolet-6700 Fourier Transform Infrared Spectroscopy system. LCMS analysis was performed on an LCMS 1260 Infinity II Agilent Technologies system (Windows 10, OpenLabs CDS Chemstation Software, 6120 Quadrupole LC/MS G7111B quaternary pump, G7129A Infinity II vialsampler, thermostated column compartment, G7117C 1260 diode array detector) with an LC Kinetex column 2.6 µm C18 (50 x 3 mm). Runs were performed at a flow-rate of 1 mL/min with a run-time of 5 min, and a solvent gradient of 0-100% MeCN in water, containing 0.1% formic acid.

Photophysical Characterization

All measurements were performed in the dark or under red light. Samples were stored at –20 °C. UV-Vis spectroscopy was performed using a Cary 60 UV-Vis Spectrophotometer equipped with a PCB 1500 High Performance Peltier thermostat (Agilent Technologies, Santa Clara, CA). Samples were measured using ultra-micro cuvettes with a 10 mm light path (BrandTech, Essex, CT). Irradiation was performed using an Optoscan Monochromator equipped with an Optosource High Intensity Arc Lamp (Cairn Research, Kent, UK) set to 15 nm full width at half maximum and installed with a 75 W UXL-S50A xenon arc lamp (USHIO, Tokyo, JP). The monochromator was controlled using a custom MATLAB program and data were analyzed and plotted using Prism 8 (GraphPad, San Diego, CA). LC-MS analysis was performed using a 1260 Infinity II LC-MS system (Windows 10, OpenLabs CDS Chemstation Software, 6120 Quadrupole LC/MS G7111B quaternary pump, G7129A Infinity II vialsampler, thermostatted column compartment, G7117C 1260 diode array detector, Agilent Technologies, Santa Clara, CA) with an LC Kinetex column (2.6 um C18, 50 x 3 mm, Phenomenex, Torrance, CA). Runs were performed at a flow rate of 1 mL/min with a runtime of 5 min, and a solvent gradient of 0–100% MeCN in water, containing 0.1% formic acid.

Thermal Relaxation

A 10 mM stock solution of fully dark-adapted compound was prepared in DMSO and diluted to 20 uM (10% DMSO in PBS; or PBS). A sample (1 mL) of the solution (20 uM) was transferred to a cuvette and placed in the UV VIS set to 37 °C. The sample was subjected to 10 min irradiation with 365 nm and absorbance at 340 nm was detected over time. Thermal half-life of the cis-isomer was determined by one-phase exponential decay.

PSS Quantification

Dark adapted samples (100% trans, 20 uM in respective solvent mixture) were irradiated with the respective wavelength for 10 min under ambient conditions. Analytical samples were prepared in Amber vials, and immediately subjected to LCMS separation (10 uL injection volume). The relative ratios of (Z)- and (E)-isomers were determined by detection at the isosbestic points at the respective elution time solvent mixtures (242, 273 nm) and the integration of the UV-absorbances was averaged.

Cell culture, molecular cloning, and gene expression

HEK293T cells were purchased from ATCC (CRL-11268), authenticated by Bio-Synthesis, Inc. and tested negative for mycoplasma using a kit from Molecular Probes. Cells were maintained in DMEM (GIBCO) supplemented with 5% fetal bovine serum and passaged by trypsin/EDTA digestion upon reaching 95% confluency. The cells were seeded 24 prior to transfection on poly-L-lysine-coated glass coverslips in a 12-well plate. Lipofectamine 2000 (Thermo Fisher) was used for transfection of DNA plasmids. For calcium imaging experiments, cells were transfected with the (human) 5-HTR of interest and GCaMP6f, in a 7:1 ratio with 0.7 µg of receptor per well. The SNAP-5-HT_{2A}R clone was made using Gibson assembly (Gibson et al., 2009) and contains the rat mGluR5 signal sequence followed by an HA-tag, a SNAP-tag and wild-type rat 5-HT_{2A}R.

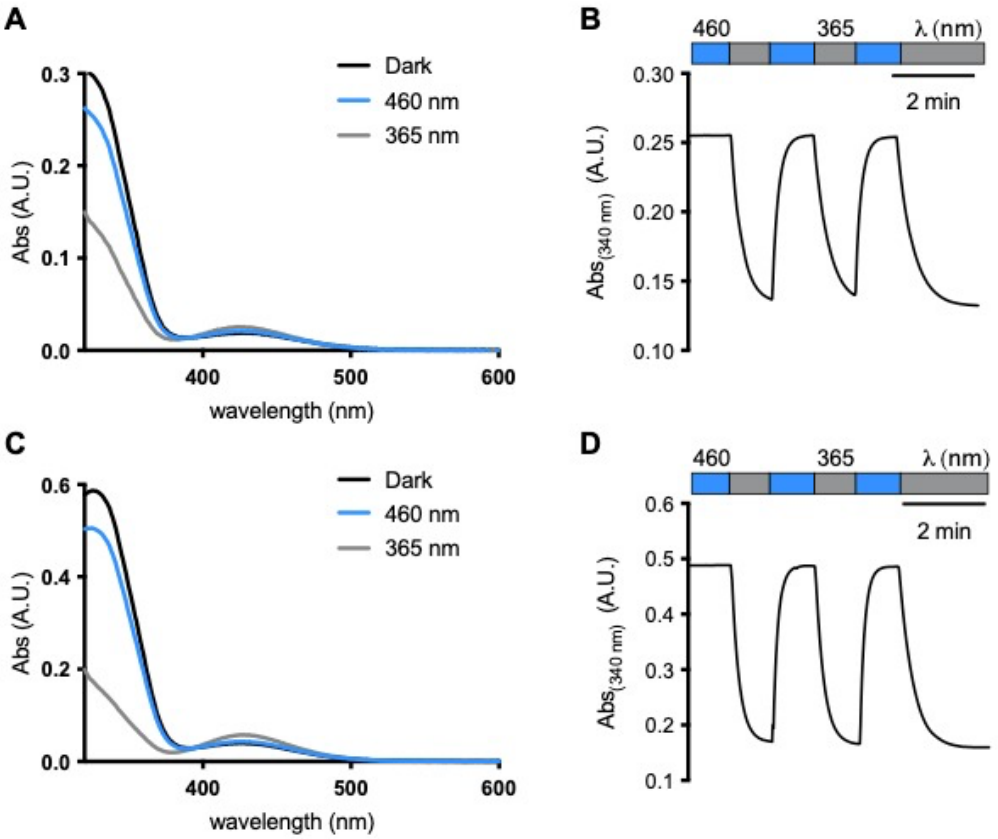
Fluorescence imaging

24–48 hr after transfection, cells were imaged at room temperature in extracellular solution containing (in mM): 135 NaCl, 5.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4. Calcium imaging experiments were conducted with continuous gravity-driven perfusion on an inverted microscope (Olympus IX73) using a 20x objective. During experiments, GCaMP6f was excited using a 488 nm laser and movies were recorded with an scMOS camera (Hamamatsu ORCA-Flash4v3.0) at 0.5 Hz with a 50 ms exposure time. Photoactivation was performed using 385 nm LED illumination (coolLED PE-4000) at 0.7 mW/mm^2 for 500-1200 ms between each 488 nm imaging frame, as previously described.[1] For PORTL experiments, cells were incubated in 10 µM of each PORTL for 45 min at 37° prior to the experiment. Ligand responses were quantified relative to the response to saturating 5-HT (1 µM). For PORTL analysis only cells which showed a clear 385 nm response were quantified. Regions of interest were selected in Olympus cellSens software and represent single cells. Data analysis was performed in RStudio, where intensities were normalized to the baseline prior to drug application. Dose response curves were fit using Prism (Graphpad). For PORTL labeing competition assay, after PORTL labeling cells were incubated for 45 min at 37° with 1 µM BG-Alexa-546 (NEB). Clusters of cells were used for analysis of fluorescence and background subtraction was done using fluorescence measurements of coverslip areas without cells.

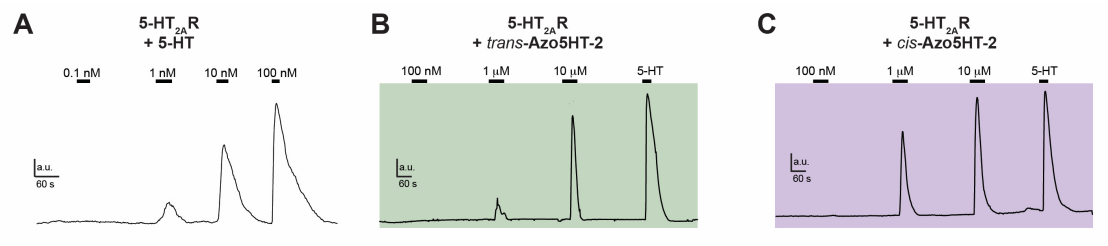
Ligand Docking

Ligand preparation and docking were done using Schrödinger's Suite Release 2020-4. The 3D structures of serotonin (5-HT), *cis*-Azo5HT-2 and *trans*-Azo5HT-2-cis-trans were manually built with Schrödinger's Maestro Version 12.6.149 and processed using LigPrep.[2] Protonation states were assigned using Epik[3] with a target pH of 7.0 ± 2.0. Geometry was optimized with the Optimized Potentials for Liquid Simulations (OPLS)-2005 force field.[4] The molecular structure of LSD-bound 5-HT_{2A} (PDB: 6WGT)[5] was retrieved from Protein Data Bank (PDB) with a resolution of 3.4 Å. The binding pockets of the three chains were very similar (0.491 Å all atom RMSD) using residues within 10 Å of the LSD. We used Chain A for all subsequent analyses. Prior to docking, the protein was prepared using Protein Preparation Wizard.[6] Stabilizing molecules were deleted, disulfides bonds created, and missing side chains were filled in using Prime.[7,8] Energy minimization was performed using the default constraint of 0.3 Å RMSD and OLPS-2005 force field.

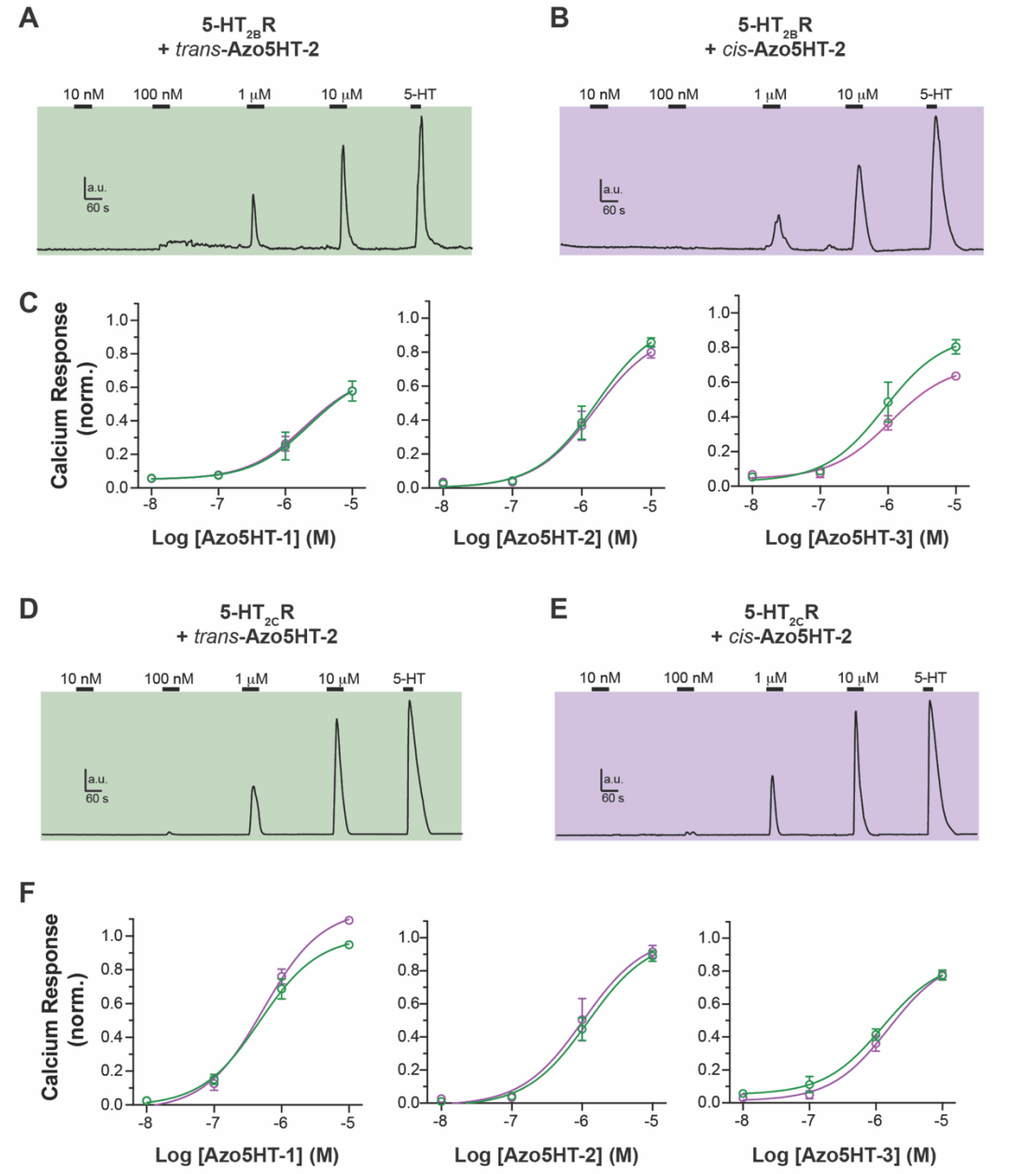
The Induced Fit Docking (IFD)[9–11] protocol was composed of 3 steps: ligands were docked with Glide, then Prime Refinement was used to allow the receptor to relax, and then the ligands were redocked into the relaxed receptor with Glide. Following Schrödinger's protocols for constraining ligand dihedral angles (to prevent the *cis* state molecule from relaxing into the lower energy *trans* state), initial Glide docking was carried out outside of the IFD script for each ligand. Receptor grid generation was carried out with a scaling factor of 1.0, using the crystal structure ligand as the center of the grid. A Van der Waals scaling factor of 0.8 was used, and SER 242 and SER 239 were selected as rotatable groups. Sampling of nitrogen inversions and ring conformations were allowed. Enhanced planarity of conjugated pi groups was selected. For the *cis* condition, constraints were placed on the CN=NC bond of the azobenzene, locking the molecule in the *cis* state. All result Glide poses were then passed to the IFD protocol for the Prime refinement and final Glide steps. For Prime, ligand sample ring conformations were allowed with an energy window of 2.5 kcal/mol. The canonical binding pose for serotonin was defined by prior studies[12,13] and comparison to the serotonin-5-HT_{1A} bound to G_i (PBD 7E2Y). In this structure, the protonated nitrogen of 5-HT forms a H-Bond with D155 (D3.32), and the indole body form a pi-pi interaction with the aromatic cluster residue F340 (F6.52). Docking poses that were determined to contain these interactions (using Maestro's protein-ligand interaction definitions) were labeled as canonical.



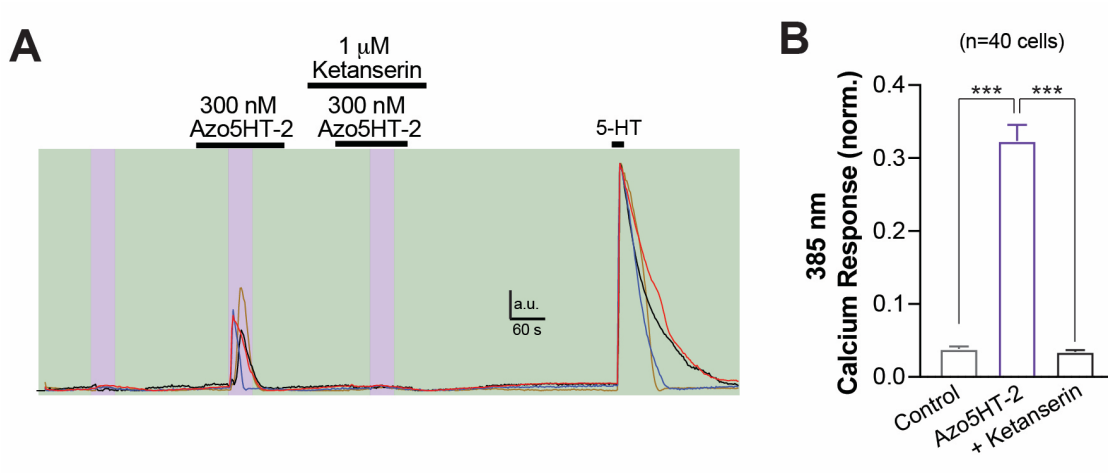
Supplementary Figure 1. Photophysical characterization of photoswitchable 5-HT derivatives **Azo5HT-1** and **Azo5HT-3**. (A) The UV-Vis spectra of **Azo5HT-1** in the dark-adapted (black, *trans*), 365 nm adapted (grey, *cis*) and 460 nm adapted (blue, *trans*) photostationary states (50 μM, DMSO). (B) Reversible cycling between **Azo5HT-1** photoisomers with alternating illumination at 365/460 nm (50 μM, DMSO). (C) The UV-Vis spectra of **Azo5HT-3** in the dark-adapted (black, *trans*), 365 nm adapted (grey, *cis*) and 460 nm adapted (blue, *trans*) photostationary states (50 μM, DMSO). (D) Reversible cycling between **Azo5HT-3** photoisomers with alternating illumination at 365/460 nm (50 μM, DMSO).



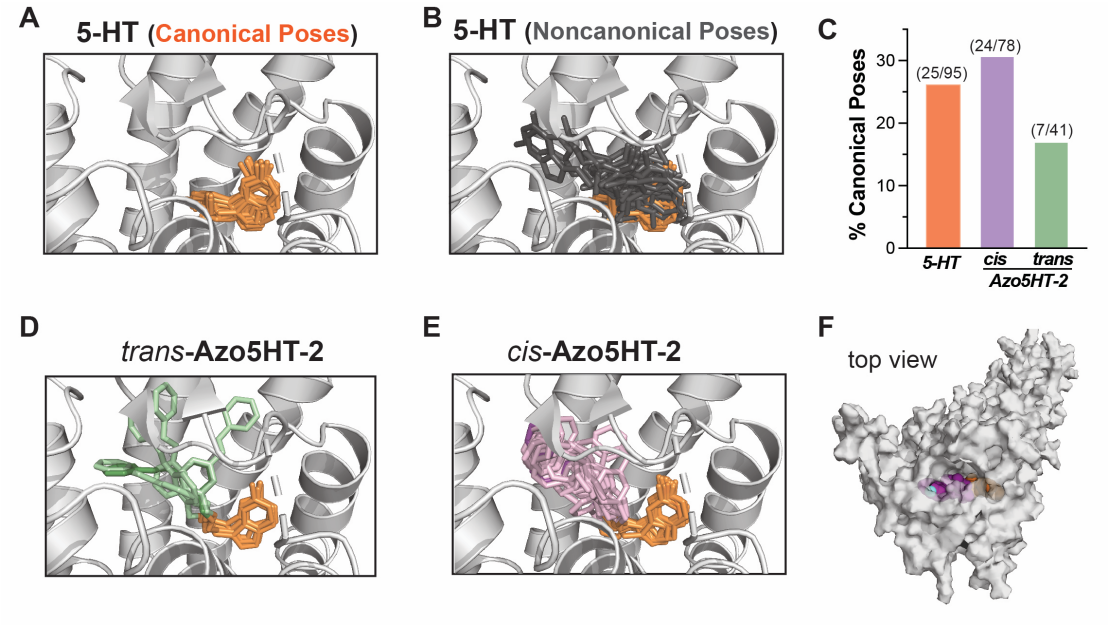
Supplementary Figure 2. Representative 5HT (A) and **Azo5HT-2** titrations (B, C) for 5-HT_{2A}R with **Azo5HT-2** showing a left-ward shift when data is collected with 385 nm illumination (C). Representative traces come from an average of 20-30 cells from a single movie. 5-HT is applied at 1 μM for normalization.



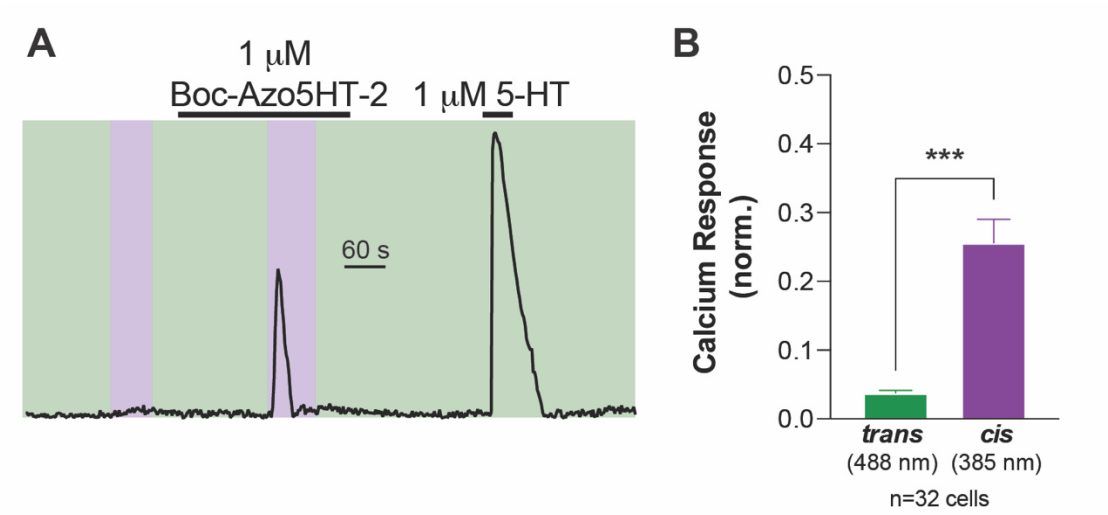
Supplementary Figure 3. Representative traces (A, B) and Dose response curves (C) for 5-HT$_{2B}$R showing no clear difference for *cis* versus *trans* **Azo5HT** compounds. Representative traces (D, E) and Dose response curves (F) for 5-HT$_{2C}$R showing no clear difference for *cis* versus *trans* **Azo5HT** compounds. Representative traces come from an average of 20-30 cells from a single movie. 5-HT is applied at 1 μM for normalization.



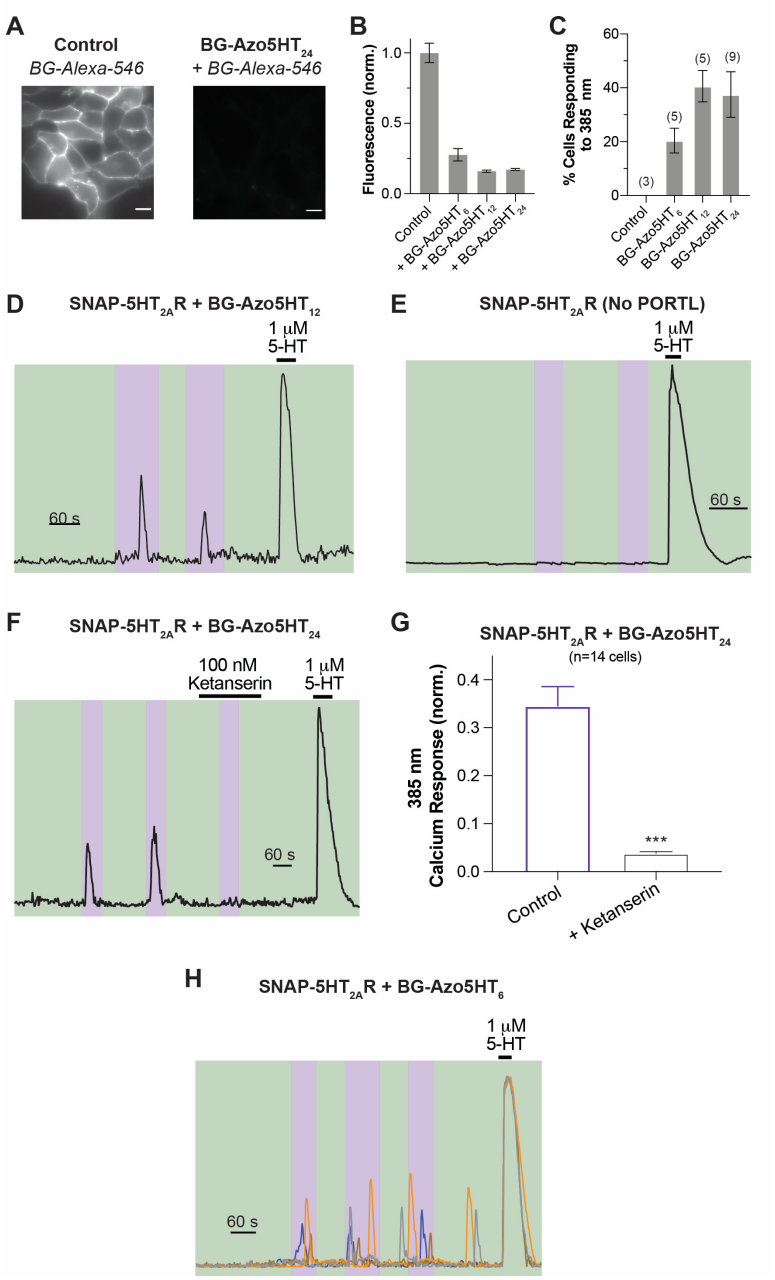
Supplementary Figure 4. Representative traces (A) and summary bar graph showing light-dependent activation of 5-HT_{2A}R by **Azo5HT-2** and block by ketanserin. Control bar shows the response to 385 nm prior to **Azo5HT-2** application, purple bar indicates the response in the presence of **Azo5HT-2** and black bars represent the response in the presence of **Azo5HT-2** and ketanserin. *** indicates statistical significance (paired T-test; $p<0.001$).



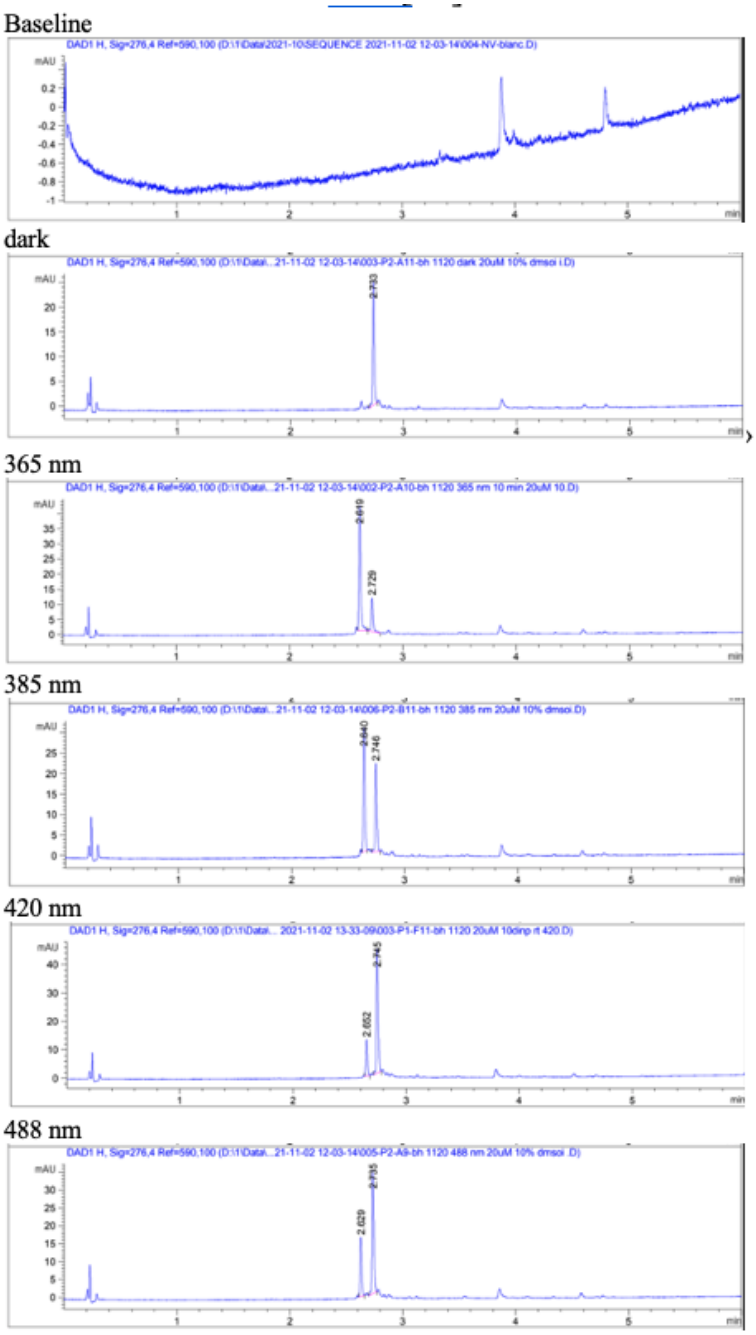
Supplementary Figure 5. Docking analysis showing canonical (A) and non-canonical 5-HT poses (B, gray molecules). (C) Summary of the proportion of poses showing canonical positioning of the 5-HT moiety. Distinct poses of *trans*-**Azo5HT-2** (D) and *cis*-**Azo5HT-2** (E) on 5-HT_{2A}R. Top view with surface representation shows potential accessibility of *cis*-**Azo5HT-2** from the extracellular surface of the receptor (F).



Supplementary Figure 6. Representative trace (A) and summary bar graph showing light-dependent activation of 5-HT_{2A}R by **Boc-Azo5HT-2**. Purple bars indicate the response to 385 nm light (*cis*) and green bars represent the response to direct application of the drug in the presence of 488 nm imaging light (*trans*). *** indicates statistical significance (paired T-test; $p<0.001$).

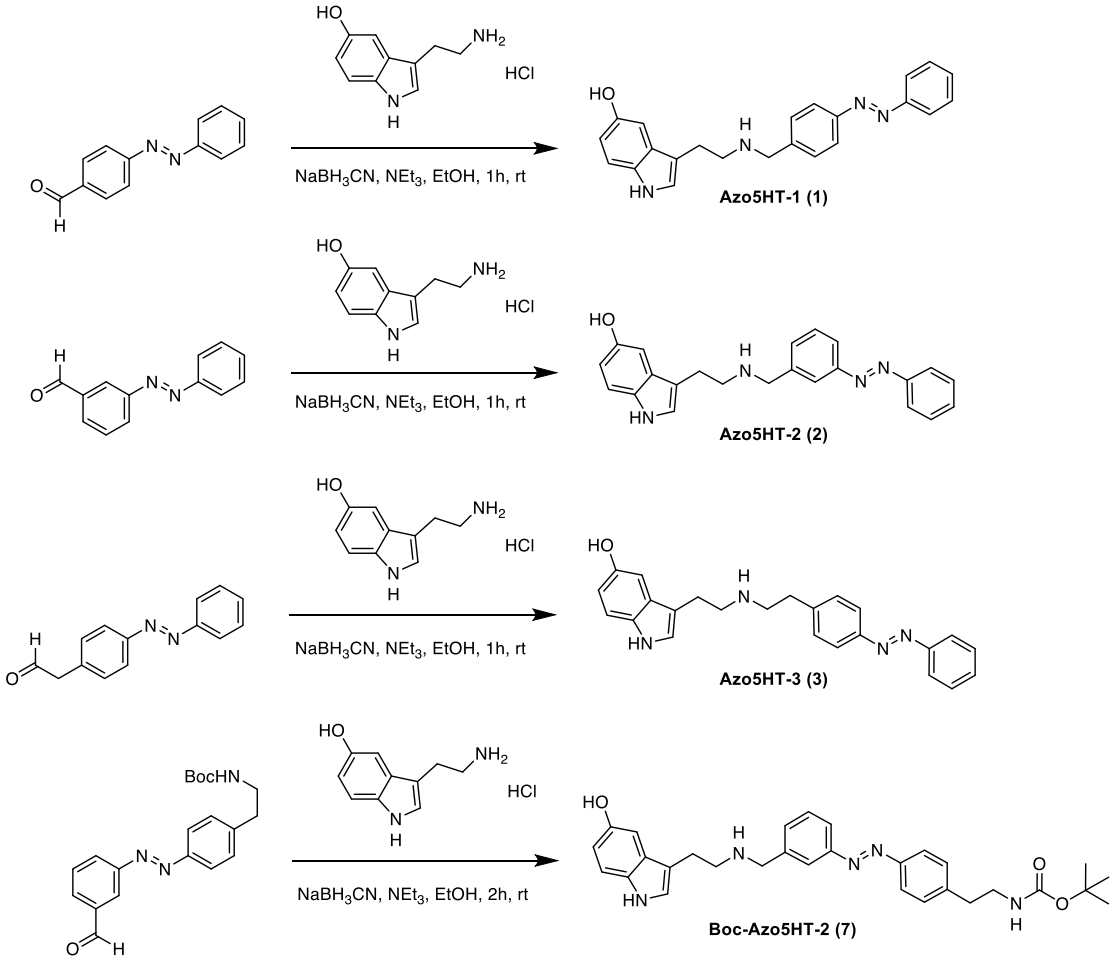


Supplementary Figure 7. (A-B) Representative images (A) and summary bar graph (B) showing that PORTL incubation dramatically decreases BG-conjugated fluorophore labeling of SNAP-5HT$_{2A}$R. This indicates that PORTL labeling is efficient and comparable across different lengths. (C) Summary of analysis oft he proportion of cells showing 385 nm light responses for controls (no PORTL labeling) or each version of **BG-Azo5HT$_n$**. The number of separate experiments is shown in parentheses. (D-G) Representative traces of individual cells showing photoactivation of SNAP-5HT$_{2A}$R by **BG-Azo5HT$_{12}$** (D), lack of light responses in no PORTL controls (E), ketanserin-sensitivity of **BG-Azo5HT$_{24}$** photoactivation (F, G), and apparent *cis* and *trans*-activation by **BG-Azo5HT$_6$** (H).*** $p<0.001$, paired t-test.

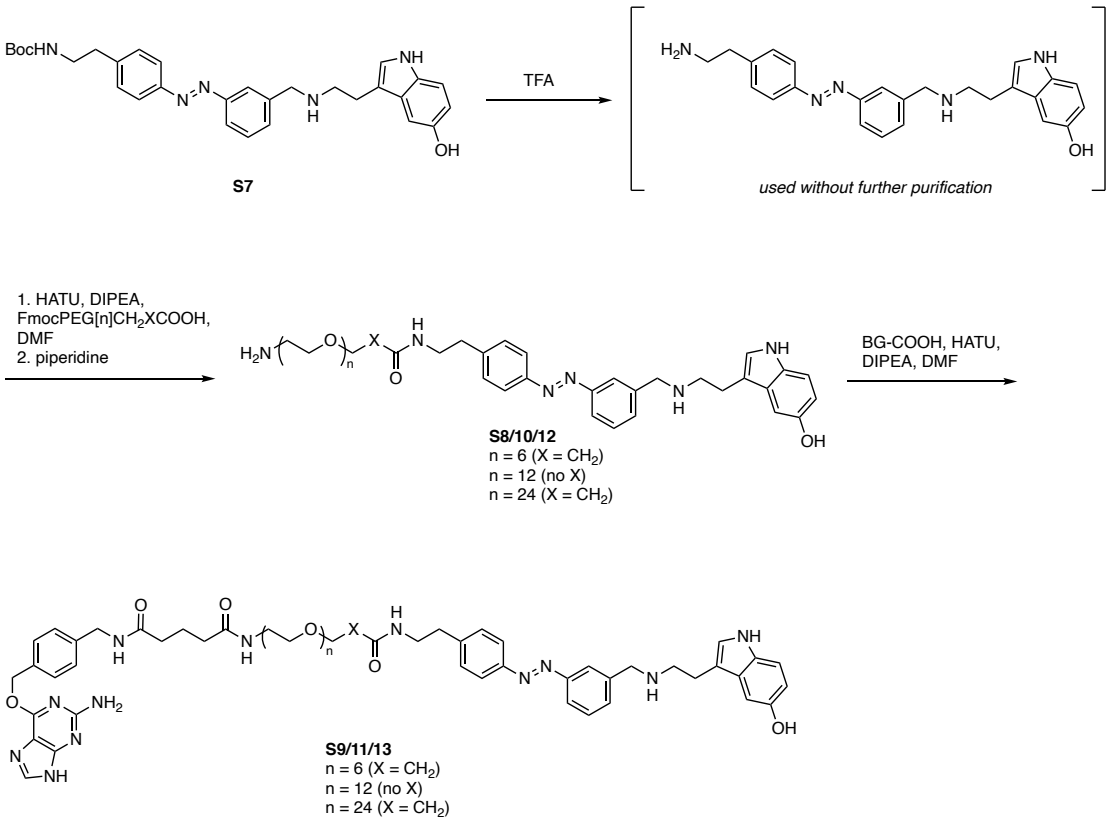


Supplementary Figure 8. Light-dependent LCMS traces of **BG-Azo5HT$_{24}$**. Compound was dissolved at 20 μM concentration in PBS with 10 %DMSO, irradiated for 10 minutes at the indicated wavelength and injected.

Synthetic Schemes



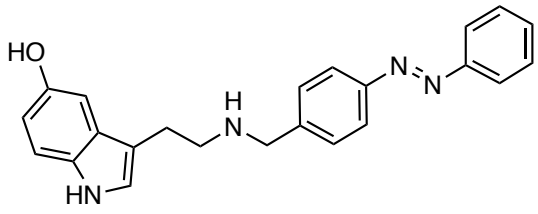
Supplementary Scheme 1. Synthesis of **Azo5HTs** and tether model **Boc-Azo5HT-2**.



Supplementary Scheme 2. Synthesis of **BGAzo5HT-PEG[n]s**.

Synthetic Details

Azo5HT-1 (S1): (*E*)-3-(2-((4-(phenyldiazenyl)benzyl)amino)ethyl)-1*H*-indol-5-ol



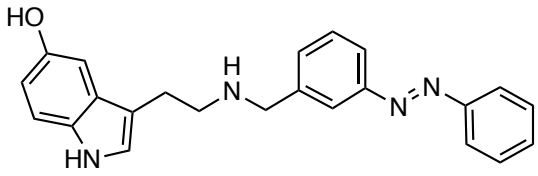
Serotonin hydrochloride (20.0 mg, 94.0 μmol, 1.0 equiv.), (*E*)-4-(phenyldiazenyl)-benzaldehyde (19.8 mg, 94.0 μmol, 1.0 equiv.), and triethylamine (19.0 mg, 188 μmol, 2.0 equiv.) were dissolved in ethanol (2.0 mL) and stirred for 10 min at room temperature. $NaBH_3CN$ (11.8 mg, 188 μmol, 2.0 equiv.) was added and the mixture was stirred for 1 h at room temperature. EtOH was removed under reduced pressure and the mixture was purified by flash column chromatography (CH_2Cl_2 to 20% MeOH in CH_2Cl_2) to yield **Azo5HT-1 (S1)** as orange solid (3.8 mg, 10.3 μmol, 11%).

^{1}H NMR (400 MHz, MeOD) δ 7.94 – 7.84 (m, 4H), 7.58 – 7.48 (m, 3H), 7.48 – 7.43 (m, 2H), 7.17 (d, *J* = 8.7 Hz, 1H), 7.01 (s, 1H), 6.92 (d, *J* = 2.3 Hz, 1H), 6.67 (dd, *J* = 8.6, 2.3 Hz, 1H), 3.89 (s, 2H), 2.96 (s, 4H).

^{13}C NMR (100 MHz, MeOD) δ 154.0, 153.2, 151.2, 143.4, 133.2, 132.2, 130.4, 130.3, 129.3, 124.4, 123.9, 123.8, 112.7, 112.5, 112.3, 103.5, 53.7, 26.0, 10.3.

HRMS: m/z calcd. for $C_{23}H_{23}N_4O^+$ ($[M+H]^+$): 371.1866 found: 371.1863.

Azo5HT-2 (S2): (*E*)-3-(2-((3-(phenyldiazenyl)benzyl)amino)ethyl)-1*H*-indol-5-ol



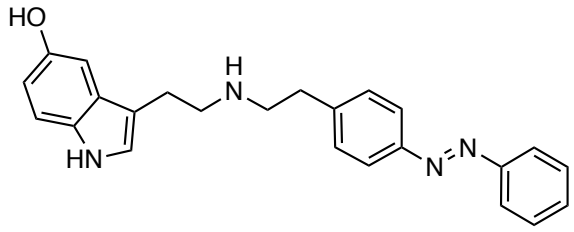
Serotonin hydrochloride (20.0 mg, 94.0 μmol, 1.0 equiv.), (*E*)-3-(phenyldiazenyl)-benzaldehyde (19.8 mg, 94.0 μmol, 1.0 equiv.), and triethylamine (19.0 mg, 188 μmol, 2.0 equiv.) were dissolved in ethanol (2.0 mL) and stirred for 10 min at room temperature. $NaBH_3CN$ (11.8 mg, 188 μmol, 2.0 equiv.) was added and the mixture was stirred for 1 h at room temperature. EtOH was removed under reduced pressure and the mixture was purified by flash column chromatography (CH_2Cl_2 to 20% MeOH in CH_2Cl_2) to yield **Azo5HT-2 (S2)** as orange solid (12.2 mg, 32.9 μmol, 35%).

^{1}H NMR (400 MHz, MeOD) δ 7.90 (dt, *J* = 8.1, 1.2 Hz, 2H), 7.85 (d, *J* = 1.8 Hz, 1H), 7.80 (dt, *J* = 7.8, 1.5 Hz, 1H), 7.58 – 7.43 (m, 4H), 7.40 (dt, *J* = 7.6, 1.5 Hz, 1H), 7.19 – 7.12 (m, 1H), 7.00 (s, 1H), 6.96 – 6.89 (m, 1H), 6.66 (dd, *J* = 8.7, 2.3 Hz, 1H), 3.88 (s, 2H), 2.94 (s, 4H).

^{13}C NMR (100 MHz, MeOD) δ 154.1, 154.0, 151.1, 141.6, 133.2, 132.3, 132.3, 130.3, 130.2, 129.3, 124.3, 123.8, 123.6, 122.9, 112.7, 112.5, 112.4, 103.5, 53.9, 50.2, 26.0.

HRMS: m/z calcd. for $C_{23}H_{23}N_4O^+$ ($[M+H]^+$): 371.1866 found: 371.1866.

Azo5HT-3 (S3): (*E*)-3-(2-((4-(phenyldiazenyl)phenethyl)amino)ethyl)-1*H*-indol-5-ol



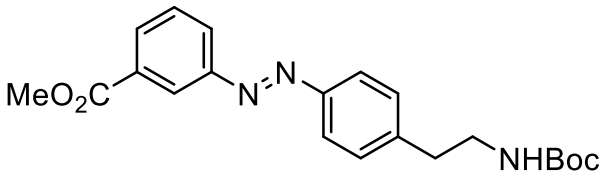
Serotonin hydrochloride (20.0 mg, 94.0 μmol, 1.0 equiv.), (*E*)-2-(4-(phenyldiazenyl)phenyl)acetaldehyde (21.1 mg, 94.0 μmol, 1.0 equiv.), and triethylamine (19.0 mg, 188 μmol, 2.0 equiv.) were dissolved in ethanol (2.0 mL) and stirred for 10 min at room temperature. $NaBH_3CN$ (11.8 mg, 188 μmol, 2.0 equiv.) was added and the mixture was stirred for 1 h at room temperature. EtOH was removed under reduced pressure and the mixture was purified by flash column chromatography (CH_2Cl_2 to 20% MeOH in CH_2Cl_2) to yield **Azo5HT-3 (S3)** as orange solid (8.8 mg, 22.9 μmol, 24%).

^{1}H NMR (400 MHz, MeOD) δ 7.95 – 7.85 (m, 2H), 7.76 – 7.70 (m, 2H), 7.58 – 7.45 (m, 3H), 7.25 – 7.19 (m, 2H), 7.16 – 7.10 (m, 1H), 6.96 – 6.90 (m, 2H), 6.67 (dd, *J* = 8.6, 2.3 Hz, 1H), 2.91 (qd, *J* = 8.5, 7.3, 2.6 Hz, 6H), 2.82 (dd, *J* = 7.9, 5.2 Hz, 2H).

^{13}C NMR (100 MHz, MeOD) δ 154.0, 152.5, 151.2, 144.5, 133.2, 132.1, 130.4, 130.2, 129.2, 124.4, 124.0, 123.7, 112.8, 112.5, 112.3, 103.4, 51.4, 50.4, 36.1, 26.0.

HRMS: m/z calcd. for $C_{24}H_{25}N_4O^+$ ($[M+H]^+$): 385.2023 found: 385.2038.

S4: methyl (E)-3-((4-(2-((tert-butoxycarbonyl)amino)ethyl)phenyl)diazenyl)benzoate



Experimental: Methyl 3-aminobenzoate (1.33 g, 8.82 mmol, 1.5 equiv.) was dissolved in CH_2Cl_2 (98 mL), treated with a solution of Oxone® (10.85 g, 17.65 mmol, 3.0 equiv.) in H_2O (98 mL) and the resulting biphasic mixture was stirred rapidly at room temperature overnight. The two phases were separated, and the organic phase was washed with 1 M HCl, saturated $NaHCO_3$ solution, and H_2O. The organic phase was dried over Na_2SO_4 and filtered. A solution of tert-butyl (4-aminophenethyl)carbamate (1.39 g, 5.88 mmol, 1.0 equiv.) in CH_2Cl_2 (20 mL) was added, followed by AcOH (98 mL). The CH_2Cl_2 was removed under reduced pressure, and the resulting solution was stirred at room temperature overnight. The AcOH was removed under reduced pressure, and the remaining oil was purified by flash column chromatography (SiO_2, gradient: 0-30% EtOAc:hexanes) to yield **S4** (1.73 g, 4.52 mmol, 75%) as an orange solid.

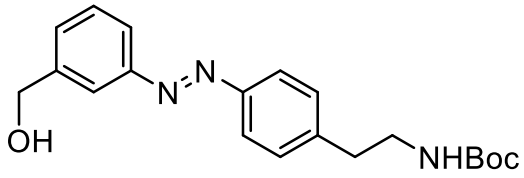
R_f = 0.77 (1:1 hexanes:EtOAc, yellow, CAN)

^{1}H NMR (400 MHz, MeOD) δ 8.5 (s, 1H), 8.1 (t, J = 8.2 Hz, 2H), 7.9 (d, J = 6.5 Hz, 2H), 7.6 (t, J = 7.8 Hz, 1H), 7.4 (d, J = 8.1 Hz, 2H), 4.0 (s, 3H), 3.4 – 3.3 (m, 2H), 2.9 (t, J = 7.3 Hz, 2H), 1.4 (s, 9H).

^{13}C NMR (101 MHz, MeOD) δ 167.8, 158.4, 153.9, 152.4, 145.1, 132.5, 132.5, 130.8, 130.5, 128.2, 124.3, 124.2, 80.0, 52.9, 42.7, 37.1, 28.8.

LCMS (ESI): calculated for $C_{21}H_{26}N_3O_4^+$ ($[M+H]^+$): 384.2, found: 384.2.

S5: tert-butyl (E)-(4-((3-(hydroxymethyl)phenyl)diazenyl)phenethyl)carbamate



Experimental: To a flame dried flask was added a solution of **S4** (1.7 g, 1.0 equiv., 4.4 mmol) and dry toluene (24 mL) and was then cooled to –78 °C. A solution of DIBAL-H (8.98 mL, 3.0 equiv., 13.2 mmol, 25 wt% in toluene) was then added dropwise. After 45 minutes, the solution was allowed to warm to rt. After 3 hours, MeOH (20 mL) was added followed by a saturated solution of aq. Rochelle's Salt. The suspension was left to stir vigorously until a bilayer was distinct. The solution was diluted with toluene, washed with H_2O and brine, and dried over Na_2SO_4 and filtered before removing the solvent under reduced pressure. The crude oil was purified by flash column chromatography (SiO_2, gradient: 0-50% EtOAc:hexanes) to yield **S5** (1.078 g, 3.03 mmol, 63% recovery) and **5** (362.0 mg, 1.02 mmol, 23%) as an orange solid.

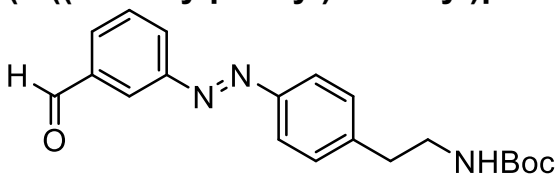
$\mathbf{R_f}$ = 0.48 (5% MeOH in DCM, yellow, VIS)

^{1}H NMR (400 MHz, MeOD) δ 7.6 – 7.5 (m, 2H), 7.4 (d, J = 8.1 Hz, 2H), 2.9 (t, J = 7.3 Hz, 2H), 7.9 – 7.8 (m, 4H), 4.7 (s, 2H), 3.4 – 3.3 (m, 2H), 1.4 (s, 9H).

^{13}C NMR (101 MHz, MeOD) δ 157.1, 152.8, 151.2, 143.2, 142.9, 129.4, 129.0, 128.8, 122.5, 121.4, 120.3, 78.6, 63.3, 41.3, 35.7, 27.3.

LCMS (ESI): calculated for $C_{20}H_{26}N_3O_3^+$ ($[M+H]^+$): 356.2, found: 356.2.

S6: tert-butyl (E)-(4-((3-formylphenyl)diazenyl)phenethyl)carbamate



Experimental: To a vial was added **S5** (355.0 mg, 0.999 mmol, 1.0 equiv.), CH_2Cl_2 (25 mL), and H_2O (1.0 mL). DMP (551 mg, 1.3 equiv., 1.30 mmol) was added, and the resultant cloudy reaction mixture was stirred vigorously for 2 hours. The mixture was diluted with ether, then concentrated under reduced pressure. The residue was taken up in Et_2O, and then washed with a mixture of 50% $Na_2S_2O_3$ (sat. aq.) and 50% $NaHCO_3$ (sat. aq.), followed by H_2O and brine. The aqueous washings were back-extracted with 20 mL of ether, and this organic layer was washed with H_2O and brine. The combined organic layers were dried with Na_2SO_4 and concentrated under reduced pressure. The crude oil was purified by flash column chromatography (SiO_2, gradient: 0-25% EtOAc:hexanes) to yield **S6** (314.5 mg, 0.890 mmol, 89%) as an orange solid.

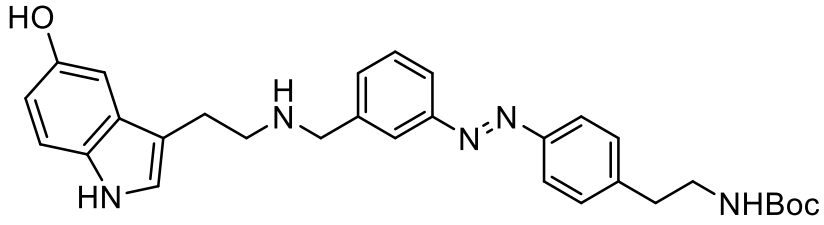
$\mathbf{R_f}$ = 0.71 (1:1 hexanes:EtOAc, yellow, CAN)

^{1}H NMR (400 MHz, MeOD) δ 10.1 (s, 1H), 8.4 (d, J = 1.8 Hz, 1H), 8.2 – 8.1 (m, 1H), 8.1 – 8.0 (m, 1H), 7.9 – 7.8 (m, 2H), 7.7 (t, J = 7.8 Hz, 1H), 7.5 – 7.3 (m, 2H), 3.4 – 3.3 (m, 2H), 2.9 – 2.8 (m, 2H), 1.4 (s, 9H).

^{13}C NMR (101 MHz, MeOD) δ 192.1, 157.0, 152.9, 151.0, 137.6, 131.0, 129.8, 129.5, 128.0, 122.8, 122.6, 119.9, 78.6, 41.3, 35.7, 27.4.

LCMS (ESI): calculated for $C_{20}H_{23}N_3O_3Na^+$ ($[M+Na]^+$): 376.2, found: 376.2.

Boc-Azo5HT-2 (S7): tert-butyl (E)-(4-((3-(((2-(5-hydroxy-1H-indol-3-yl)ethyl)amino)methyl)phenyl)diazenyl)phenethyl)carbamate



Experimental: To a flame dried flask was added **S6** (221 mg, 1.0 equiv., 0.625 mmol), serotonin hydrochloride (133 mg, 1.0 equiv., 0.625 mmol), and triethylamine (174 µL, 2.0 equiv., 1.25 mmol). The mixture was dissolved in ethanol (20 mL) and stirred for 10 min at room temperature. Sodium cyanoborohydride (78.6 mg, 2.0 equiv., 1.25 mmol) was then added and the mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure, and the crude oil was purified by flash column chromatography (SiO_2, gradient: 0-5% MeOH:CH_2Cl_2) to yield **Boc-Azo5HT-2** (**S7**) (187.1 mg, 0.364 mmol, 58%) as an orange solid.

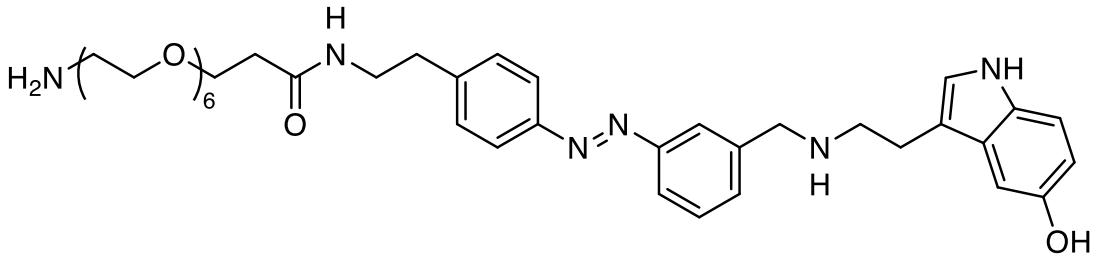
$\mathbf{R_f}$ = 0.29 (5% MeOH in DCM, $KMnO_4$)

^{1}H NMR (600 MHz, MeOD) δ 7.9 – 7.8 (m, 3H), 7.8 – 7.8 (m, 1H), 7.5 (t, J = 7.7 Hz, 1H), 7.4 – 7.3 (m, 3H), 7.2 (d, J = 8.6 Hz, 1H), 7.0 (s, 1H), 6.9 (d, J = 2.3 Hz, 1H), 6.7 (dd, J = 8.6, 2.3 Hz, 1H), 3.9 (s, 2H), 3.3 (s, 2H), 3.0 – 2.9 (m, 4H), 2.8 (t, J = 7.2 Hz, 2H), 1.4 (s, 9H).

^{13}C NMR (151 MHz, MeOD) δ 157.0, 152.8, 151.1, 149.8, 143.2, 139.4, 131.8, 130.8, 129.4, 129.0, 127.9, 123.0, 122.6, 122.3, 121.7, 111.4, 111.1, 110.7, 102.1, 78.6, 52.3, 48.7, 41.3, 35.7, 27.4, 24.4.

LCMS (ESI): calculated for $C_{29}H_{33}N_2O_5^+$ ($[M+H]^+$): 514.3, found: 514.2.

Azo5HT-PEG[6] (S8): (E)-1-amino-N-(4-((3-(((2-(5-hydroxy-1H-indol-3-yl)ethyl)amino)methyl)phenyl)diazenyl)phenethyl)-3,6,9,12,15,18-hexaoxahenicosan-21-amide



Experimental: In a 4 mL glass vial, **S7** (8.7 mg, 16.9 µmol, 1.0 equiv.) was treated with TFA (1 mL). After stirring for 1 min at ambient temperature, the TFA was removed under a gentle stream of nitrogen. The remaining crude was taken up in MeOH and the solvent removed again, and LCMS showed full deprotection. The crude was dissolved in DMF (0.2 mL), and FmocNHPEG[6]CH2CH2COOH (7.8 mg, 13.6 µmol, 0.8 equiv., as solution in 300 µL DMF) and DIPEA (15 µL, 87.4 µmol, 5.0 equiv.) were added. Then, HATU (5.2 mg, 13.6 µmol, 0.8 equiv.) was added in one portion and the reaction mixture was stirred for 30 min, until conversion was determined by LCMS. Piperidine (25 µL, 254.1 µmol, 15 equiv.) was added and the solution stirred another 30 min, until deprotection was determined by LCMS. The solution was treated with AcOH under slight evolution of fumes, and then subjected to RP-HPLC purification (5–43% MeCN in H_2O with 0.1% formic acid over 7 min, semi-preparative column, 8.0 mL/min flow rate, detection at 360 nm, t_R = 4.239 min) to yield **S8** after lyophilization a yellow oil in 26% yield (3.3 mg, 4.4 µmol).

Note: If more than one equivalent of acid-coupling partner is used, the secondary amine engages in peptide coupling as well. This applies to all further amid coupling reactions.

LCMS (SM Azo-NH-Boc, 5-100% MeCN in H_2O with 0.1% formic acid over 5 min) t_R = 3.226 min, 360 nm detection.

LRMS (ESI, SM Azo-NH-Boc ESI): calc. for $C_{30}H_{36}N_5O_3^+$ $[M+H]^+$: 513.3; found 513.3.

LCMS (intermediate Azo-NH_2, 5-100% MeCN in H_2O with 0.1% formic acid over 5 min) t_R = 2.106 min, 360 nm detection.

LRMS (ESI, intermediate Azo-NH_2): calc. for $C_{25}H_{28}N_5O^+$ $[M+H]^+$: 414.2; found 414.2.

LCMS (intermediate NHFmoc, 5-100% MeCN in H_2O with 0.1% formic acid over 5 min) t_R = 3.420 min, 360 nm detection.

LRMS (ESI, intermediate amide NHFmoc): calc. $C_{55}H_{68}N_6O_{10}^{2+}$ $[M+2H]^{2+}$: 486.5; found 486.3.

Product

LCMS (5-100% MeCN in H_2O with 0.1% formic acid over 5 min) t_R = 2.341 min, 360 nm detection.

LRMS (ESI): calc. for $C_{40}H_{58}N_6O_8^{2+}$ $[M+2H]^{2+}$: 375.2; found 375.3.

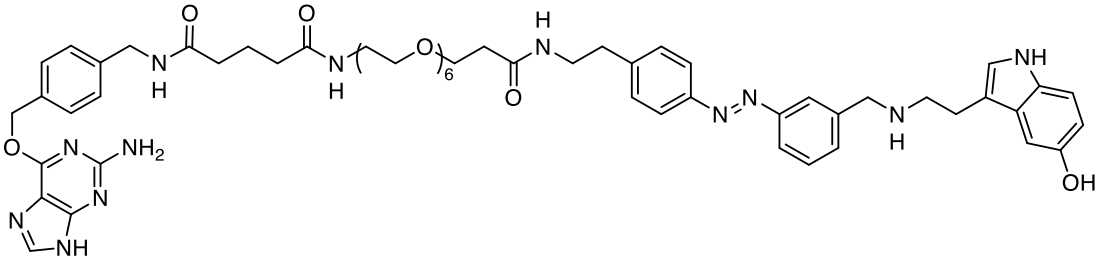
HRMS (ESI): calc. for $C_{40}H_{58}N_6O_8^{2+}$ $[M+2H]^{2+}$: 375.7168; found 375.7155.

^{1}H NMR (400 MHz, MeOD) δ 8.1 – 7.8 (m, 4H), 7.7 – 7.5 (m, 2H), 7.4 (d, J = 8.3 Hz, 2H), 7.2 (d, J = 8.7 Hz, 1H), 7.1 (s, 1H), 6.9 (d, J = 2.4 Hz, 1H), 6.7 (dd, J = 8.6, 2.4 Hz, 1H), 4.3 (s, 2H), 3.8 – 3.4 (m, 28H), 3.2 – 3.1 (m, 4H), 2.9 (d, J = 7.2 Hz, 2H), 2.5 (t, J = 6.0 Hz, 2H).

^{13}C NMR (101 MHz, MeOD) δ 174.0, 154.3, 152.5, 151.5, 144.9, 133.2, 131.1, 130.9, 124.9, 124.8, 124.5, 124.1, 113.0, 112.8, 103.2, 71.4, 71.4, 71.3, 71.2, 71.2, 71.0, 70.7, 68.3, 68.0, 52.2, 41.6, 40.6, 37.4, 36.4, 23.8.

FTIR (neat) v = 3401 (b), 2918 (w), 1679 (s), 1598 (s), 1457 (w), 1352 (w), 1294 (w), 1202 (s), 1180 (w), 1107 (s), 834 (w), 800 (w).

BGAzo5HT-PEG[6] (S9): (E)-N1-(4-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl)-N5-(24-(4-((3-(((2-(5-hydroxy-1H-indol-3-yl)ethyl)amino)methyl)phenyl)diazenyl)phenyl)-21-oxo-3,6,9,12,15,18-hexaoxa-22-azatetracosyl)glutaramide

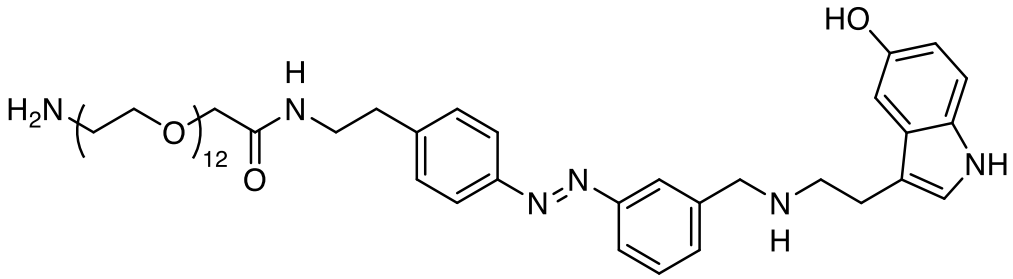


Experimental: In a 4 mL vial, 5-((4-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl) amino)-5-oxopentanoic acid (BG-COOH)[14] (1.8 mg, 4.7 µmol, 0.9 eq) and **S8** (3.9 mg, 5.2 µmol, 1.0 equiv.) were dissolved in DMF (100 µL). HATU (1.8 mg, 4.7 µmol, 0.9 equiv.) and DIPEA (10 µL, 0.057 mmol, 35.0 eq) were added and the light orange reaction mixture was stirred for 30 min, until conversion was determined by LCMS. The mixture was diluted with MeCN, treated with AcOH (20 µL) and subjected to RP-HPLC purification (semiprep column, 5-35% MeCN in H_2O with 0.1% formic acid over 10 min, semi-preparative column, 9.0 mL/min flow rate, detection at 360 nm, t_R = 9.384 min). After lyophilization, the obtained product was subjected to another hplc purification under equal conditions, to remove very close-eluting byproducts. After lyophilization, **S9** was obtained as a yellow oil in 19% yield (1.1 mg, 0.986 µmol).

LCMS (5-100% MeCN in H_2O with 0.1% formic acid over 5 min) t_R = 2.616 min, 360 nm detection.
LRMS (ESI): calc. for $C_{58}H_{76}N_{12}O_{11}^{2+}$ $[M+2H]^{2+}$: 558.3; found 558.4.
HRMS (ESI): calc. for $C_{58}H_{76}N_{12}O_{11}^{2+}$ $[M+2H]^{2+}$: 558.2871; found 558.2852.

^{1}H NMR (400 MHz, MeOD) δ 8.0 – 7.8 (m, 5H), 7.7 – 7.5 (m, 2H), 7.5 – 7.4 (m, 4H), 7.3 (d, J = 7.6 Hz, 2H), 7.2 (d, J = 8.7 Hz, 1H), 7.1 (s, 1H), 7.0 – 6.9 (m, 1H), 6.7 (dd, J = 8.7, 2.3 Hz, 1H), 5.5 (s, 2H), 4.4 (s, 2H), 4.3 (s, 2H), 3.7 (t, J = 6.1 Hz, 2H), 3.6 – 3.6 (m, 4H), 3.6 – 3.6 (m, 4H), 3.6 – 3.5 (m, 20H), 3.2 – 3.1 (m, 2H), 2.9 (t, J = 6.9 Hz, 2H), 2.4 (t, J = 6.0 Hz, 2H), 2.2 (dt, J = 14.9, 7.4 Hz, 4H), 1.9 (t, J = 7.4 Hz, 2H).
^{13}C NMR (101 MHz, MeOD) δ 175.2, 175.2, 174.1, 154.3, 152.5, 151.5, 145.0, 140.1, 137.4, 136.9, 133.1, 130.9, 129.7, 128.7, 124.9, 124.4, 124.1, 113.0, 112.8, 103.2, 71.5, 71.4, 71.4, 71.3, 71.2, 70.5, 68.6, 68.2, 52.2, 41.6, 40.3, 37.7, 36.4, 36.1, 23.2.

Azo5HT-PEG[12] (S10): (E)-38-amino-N-(4-((3-(((2-(5-hydroxy-1H-indol-3-yl)ethyl)amino)methyl)phenyl)diazenyl)phenethyl)-3,6,9,12,15,18,21,24,27,30,33,36-dodecaoxaoctatriacontanamide



Experimental: In a 4 mL glass vial, **Boc-Azo5HT-2** (**S7**) (11.7 mg, 22.8 µmol, 1.0 equiv.) was treated with TFA (1 mL). After stirring for 1 min at ambient temperature, the TFA was removed under a gentle stream of nitrogen. The remaining crude was taken up in MeOH and the solvent removed again, and full deprotection confirmed by LCMS. The crude oil was dissolved in DMF (0.2 mL), and FmocNHPEG[12]CH2COOH (20.7 mg, 25.1 µmol, 1.1 equiv.) and DIPEA (20 µL, 113.9 µmol, 5.0 equiv.) were added. The reaction mixture was stirred for 30 min, until conversion was determined by LCMS. Piperidine (50 µL, 505 µmol, 22 equiv.) was added and the solution stirred another 30 min, until deprotection was confirmed by LCMS. The solution was treated with AcOH under slight evolution of fumes, and then subjected to RP-HPLC purification (5-45% MeCN in H_2O with 0.1% formic acid over 7 min, semi-preparative column, 8.0 mL/min flow rate, detection at 360 nm, t_R = 4.658 min) to yield **S10** after lyophilization a yellow oil in 53% yield (12.1 mg, 12.1 µmol).

LCMS (intermediate amide NHFmoc, 5-100% MeCN in H_2O with 0.1% formic acid over 5 min) t_R = 3.495 min, 360 nm detection.

LRMS (ESI, intermediate amide NHFmoc): calc. for $C_{66}H_{90}N_6O_{16}^{2+}$ $[M+2H]^{2+}$: 611.3; found 611.4.

Product

LCMS (5-100% MeCN in H_2O with 0.1% formic acid over 5 min) t_R°=°2.478°min, 360°nm detection.

LRMS (ESI): calc. for $C_{51}H_{80}N_6O_{14}^{2+}$ $[M+2H]^{2+}$: 500.2; found 500.2.

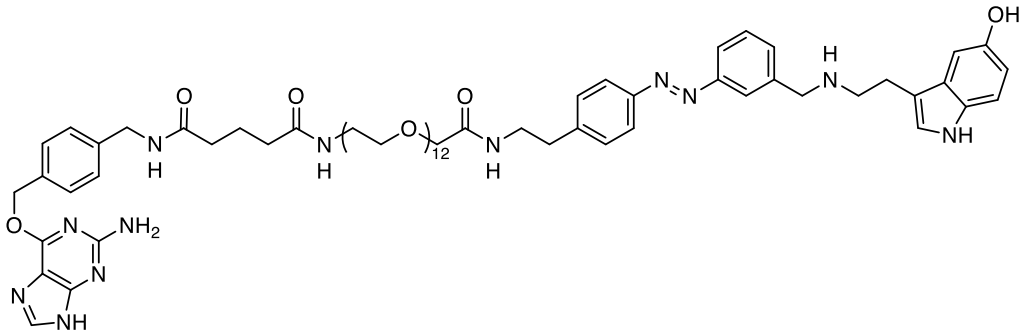
HRMS (ESI): calc. for $C_{51}H_{80}N_6O_{14}^{2+}$ $[M+2H]^{2+}$: 500.2861; found 500.2844.

^{1}H NMR (400 MHz, MeOD) δ 8.0 (s, 1H), 7.9 – 7.8 (m, 3H), 7.6 – 7.5 (m, 2H), 7.5 – 7.4 (m, 2H), 7.2 (d, J = 8.6 Hz, 1H), 7.1 (s, 1H), 6.9 (d, J = 2.3 Hz, 1H), 6.7 (dd, J = 8.7, 2.3 Hz, 1H), 4.2 (s, 2H), 4.0 (s, 2H), 3.8 – 3.7 (m, 2H), 3.7 – 3.6 (m, 50H), 3.3 – 3.2 (m, 2H), 3.2 – 3.1 (m, 2H), 3.1 (t, J = 7.5 Hz, 2H), 3.0 (t, J = 7.2 Hz, 2H).

^{13}C NMR (101 MHz, MeOD) δ 172.7, 154.3, 152.6, 151.5, 144.7, 133.2, 133.0, 131.0, 129.0, 124.8, 124.4, 124.3, 124.1, 112.9, 112.7, 110.4, 103.3, 71.9, 71.5, 71.4, 71.4, 71.3, 71.3, 71.3, 71.2, 71.2, 71.2, 71.1, 71.1, 71.0, 70.7, 68.0, 52.6, 41.1, 40.6, 36.4, 24.3.

IR (FTIR) v = 3400 (b), 2920 (w), 1594 (s), 1455 (w), 1350 (m), 1252 (w), 1101 (s), 951 (w).

BGAzo5HT-PEG[12] (S11): (E)-N1-(4-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl)-N5-(1-(4-((3-(((2-(5-hydroxy-1H-indol-3-yl)ethyl)amino)methyl)phenyl)diazenyl)phenyl)-4-oxo-6,9,12,15,18,21,24,27,30,33,36,39-dodecaoxa-3-azahentetracontan-41-yl)glutaramide:



Experimental: In a 4 mL vial, a mixture of BG-COOH (1.5 mg, 4.0 µmol, 1.0 eq), **S10** (4.0 mg, 4.0 µmol, 1.0 equiv.) and DIPEA (3 µL, 20 µmol, 5.0 eq) were dissolved in DMF (100 µL). Then, HATU (1.5 mg, 4.0 µmol, 1.0 equiv.) was added in one go and the light orange reaction mixture was stirred for 30 min, until conversion was determined by LCMS. The mixture was diluted with MeCN, treated with AcOH (20 µL) and subjected to RP-HPLC purification (semiprep column, 5-45% MeCN in H_2O with 0.1% formic acid over 10 min, semi-preparative column, 9.0 mL/min flow rate, detection at 360°nm, t_R = 6.488 min) to yield **S11** after lyophilization as a yellow oil in 40% yield (2.2 mg, 1.6 µmol).

LCMS (5-100% MeCN in H_2O with 0.1% formic acid over 5 min) t_R°=°2.692°min, 360°nm detection.

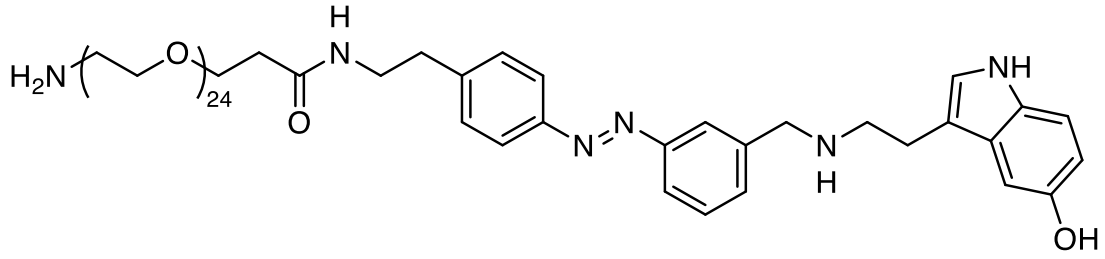
LRMS (ESI): calc. for $C_{69}H_{98}N_{12}O_{17}^{2+}$ $[M+2H]^{2+}$: 683.3; found 683.4.

HRMS (ESI): calc. for $C_{69}H_{98}N_{12}O_{17}^{2+}$ $[M+2H]^{2+}$: 683.8596; found 683.8588

^{1}H NMR (400 MHz, MeOD) δ 8.0 – 7.8 (m, 5H), 7.6 – 7.4 (m, 6H), 7.3 (d, J = 7.9 Hz, 2H), 7.2 (d, J = 8.6 Hz, 1H), 7.1 (d, J = 3.5 Hz, 1H), 6.9 (d, J = 2.2 Hz, 1H), 6.7 (dd, J = 8.6, 2.4 Hz, 1H), 5.5 (s, 2H), 4.4 (s, 2H), 4.2 (s, 2H), 4.0 (d, J = 2.6 Hz, 2H), 3.6 – 3.5 (m, 50H), 3.2 (t, J = 7.5 Hz, 2H), 3.1 (t, J = 7.5 Hz, 2H), 3.0 – 2.9 (m, 2H), 2.3 (dt, J = 14.8, 7.5 Hz, 4H), 2.0 – 1.8 (m, 2H).

^{13}C NMR (101 MHz, MeOD) δ 175.4, 175.2, 172.8, 154.3, 152.6, 151.4, 144.7, 140.1, 137.0, 133.2, 131.0, 129.7, 128.7, 124.8, 124.1, 112.9, 112.7, 103.3, 71.9, 71.5, 71.5, 71.3, 71.2, 71.1, 70.5, 68.6, 52.8, 43.8, 41.1, 40.3, 36.3, 36.2, 23.2.

Azo5HT-PEG[24] (S12): (E)-1-amino-N-(4-((3-(((2-(5-hydroxy-1H-indol-3-yl)ethyl)amino)methyl)phenyl)diazenyl)phenethyl)-3,6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72-tetracosaoxapentaheptacontan-75-amide



Experimental: In a 4 mL glass vial, **Boc-Azo5HT-2** (**S7**) (7.8 mg, 0.019 mmol, 1.0 equiv.) was treated with TFA (1 mL). After stirring for 1 min at ambient temperature, the TFA was removed under a gentle stream of nitrogen. The remaining crude was taken up in MeOH and the solvent removed again. The crude was dissolved in DMF (0.2 mL), and FmocNHPEG[24]CH2CH2COOH (25.8 mg, 0.019°mmol, 1.0°equiv.) and DIPEA (7°µL, 0.040°mmol, 5.0°equiv.) were added. Then, HATU (7.2 mg, 0.019 mmol, 1.0 equiv.) was added in one portion and the reaction mixture was stirred until full conversion was determined by LCMS. Piperidine (47°µL, 0.4729°mmol, 25°equiv.) was added and the solution stirred another 30°min. The solution was treated with AcOH under slight evolution of fumes, and then subjected to RP-HPLC purification (5-50% MeCN in H_2O with 0.1% formic acid over 14 min, semi-preparative column, 8.0 mL/min flow rate, detection at 360 nm, t_R = 7.977 min) to yield **S12** after lyophilization a yellow oil in 32% yield (9.2 mg, 0.006 mmol).

LCMS (intermediate NHFmoc, 5-100% MeCN in H_2O with 0.1% formic acid over 5 min) t_R = 3.520 min, 360 nm detection.

LRMS (ESI, intermediate NHFmoc): calc. for $C_{91}H_{140}N_6O_{28}^{2+}$ $[M+2H]^{2+}$: 882.5; found 882.7.

Product:

LCMS (5-100% MeCN in H_2O with 0.1% formic acid over 5 min) t_R = 2.776 min, 360 nm detection.

LRMS (ESI): calc. for $C_{76}H_{130}N_6O_{26}^{2+}$ $[M+2H]^{2+}$: 771.5; found 771.5.

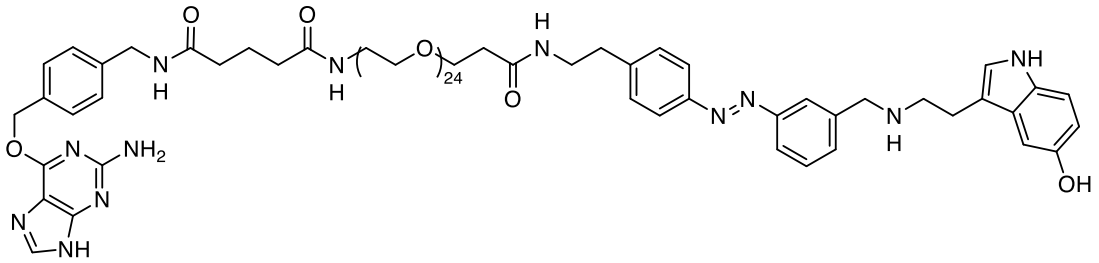
HRMS (ESI): calc. for $C_{76}H_{130}N_6O_{26}^{2+}$ $[M+2H]^{2+}$: 771.4512; found 771.4506.

^{1}H NMR (400 MHz, MeOD) δ 7.9 – 7.8 (m, 5H), 7.6 – 7.4 (m, 4H), 7.2 (d, J = 8.7 Hz, 1H), 7.1 (s, 1H), 6.9 (d, J = 2.3 Hz, 1H), 6.7 (dd, J = 8.7, 2.3 Hz, 1H), 4.0 (s, 2H), 3.8 – 3.7 (m, 2H), 3.7 – 3.6 (m, 94H), 3.5 (d, J = 7.1 Hz, 2H), 3.2 – 3.1 (m, 2H), 3.1 – 3.1 (m, 2H), 3.0 – 3.0 (m, 2H), 2.9 (t, J = 7.1 Hz, 3H), 2.4 (t, J = 6.0 Hz, 2H).

^{13}C NMR (101 MHz, MeOD) δ 174.1, 154.2, 152.5, 151.3, 144.7, 139.7, 133.1, 132.6, 130.9, 130.6, 129.2, 124.6, 124.1, 123.8, 123.6, 112.9, 112.6, 111.6, 103.4, 71.5, 71.5, 71.4, 71.4, 71.3, 71.3, 71.3, 71.3, 71.2, 71.2, 71.2, 71.1, 71.0, 70.8, 68.2, 53.4, 41.6, 40.8, 37.7, 36.3, 25.4.

FTIR (neat) v = 3269 (b), 2869 (m), 1598 (m), 1454 (w), 1348 (,), 1298 (w), 1248 (w), 1101 (s), 950 (w), 843 (m).

BGAzo5HT-PEG[24] (S13): (E)-N1-(4-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl)-N5-(78-(4-((3-(((2-(5-hydroxy-1H-indol-3-yl)ethyl)amino)methyl)phenyl)diazenyl)phenyl)-75-oxo-3,6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72-tetracosaoxa-76-azaoctaheptacontyl)glutaramide



Experimental: In a 4 mL vial, BG-COOH (2.1 mg, 5.6 µmol, 1.0 eq) and **12** (8.6 mg, 5.6 µmol, 1.0 equiv.) were dissolved in DMF (200 µL). HATU (2.1 mg, 5.6 µmol, 1.0 equiv.) and DIPEA (5 µL, 0.028 mmol, 5.0 eq) were added and the light orange reaction mixture was stirred for 30 min, until conversion was determined by LCMS. The mixture was diluted with MeCN, treated with AcOH (10 µL) and subjected to RP-HPLC purification (semiprep column, 15-50% MeCN in H_2O with 0.1% formic acid over 10 min, semi-preparative column, 9.0 mL/min flow rate, detection at 360 nm, t_R = 6.600 min (*trans*) and 5.853 (*cis*)) to yield **13** after lyophilization as a yellow oil in 30% yield (3.2°mg, 1.7°µmol).

Note: The ^{13}C-spectrum doesn't resolve the peak at 50 ppm, which is observed in HSQC.

LCMS (5-100% MeCN in H_2O with 0.1% formic acid over 5 min) t_R°=°2.748°min, 360°nm detection.

LRMS (ESI): calc. for $C_{94}H_{148}N_{12}O_{29}^{2+}$ $[M+2H]^{2+}$: 954.5; found 558.4.

HRMS (ESI): calc. for $C_{94}H_{148}N_{12}O_{29}^{2+}$ $[M+2H]^{2+}$: 954.5253; found 955.1.

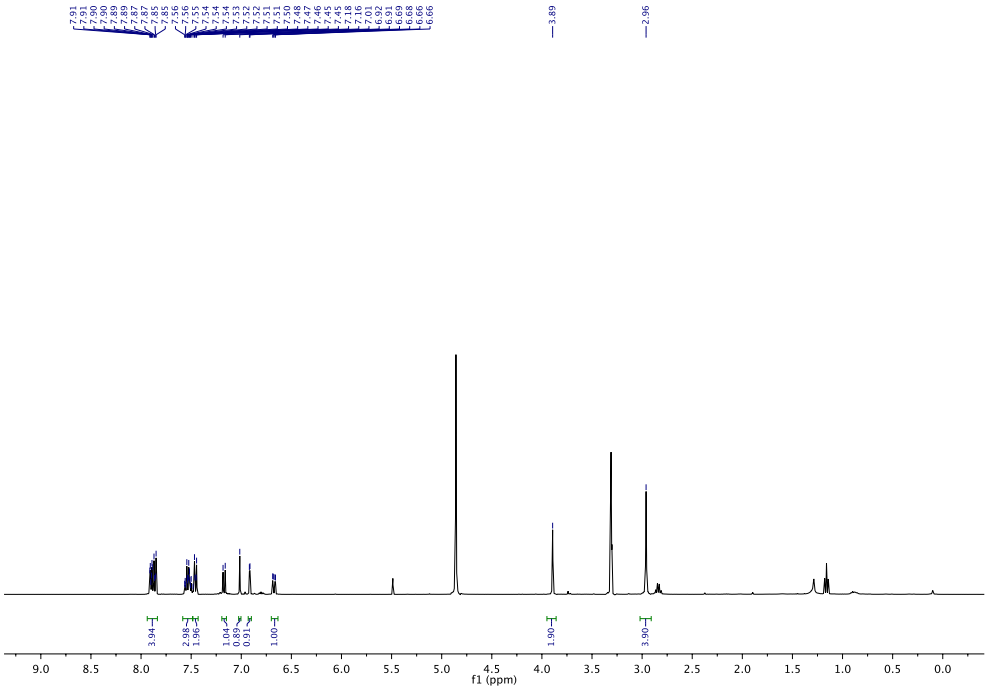
^{1}H NMR (400 MHz, MeOD) δ 8.0 – 7.8 (m, 5H), 7.7 – 7.6 (m, 2H), 7.5 – 7.4 (m, 4H), 7.4 – 7.3 (m, 2H), 7.2 (d, J = 8.8 Hz, 1H), 7.1 (s, 1H), 7.0 (d, J = 2.3 Hz, 1H), 6.7 (dd, J = 8.6, 2.1 Hz, 1H), 5.6 (s, 2H), 4.4 (s, 2H), 4.3 (s, 2H), 3.6 – 3.6 (m, 101H), 3.1 – 3.0 (m, 2H), 3.0 – 2.9 (m, 2H), 2.4 (t, J = 6.0 Hz, 2H), 2.3 – 2.2 (m, 4H), 2.0 – 1.9 (m, 2H).

^{13}C NMR (101 MHz, MeOD) δ 176.3, 175.7, 175.1, 154.3, 152.5, 151.6, 145.1, 140.1, 137.0, 134.3, 133.1, 131.2, 131.0, 129.7, 128.9, 128.7, 125.0, 124.1, 113.1, 112.9, 103.2, 71.3, 71.3, 70.5, 68.6, 68.2, 43.8, 41.6, 40.3, 37.6, 36.3, 36.2, 23.2.

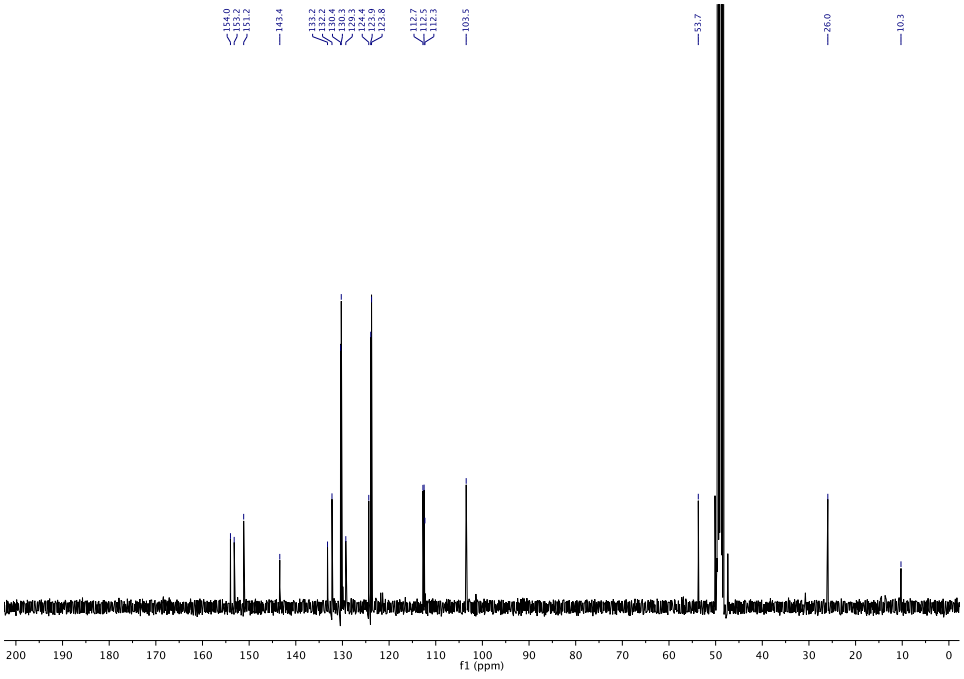
^{1}H and ^{13}C Spectra

Azo5HT-1 (S1)

^{1}H NMR

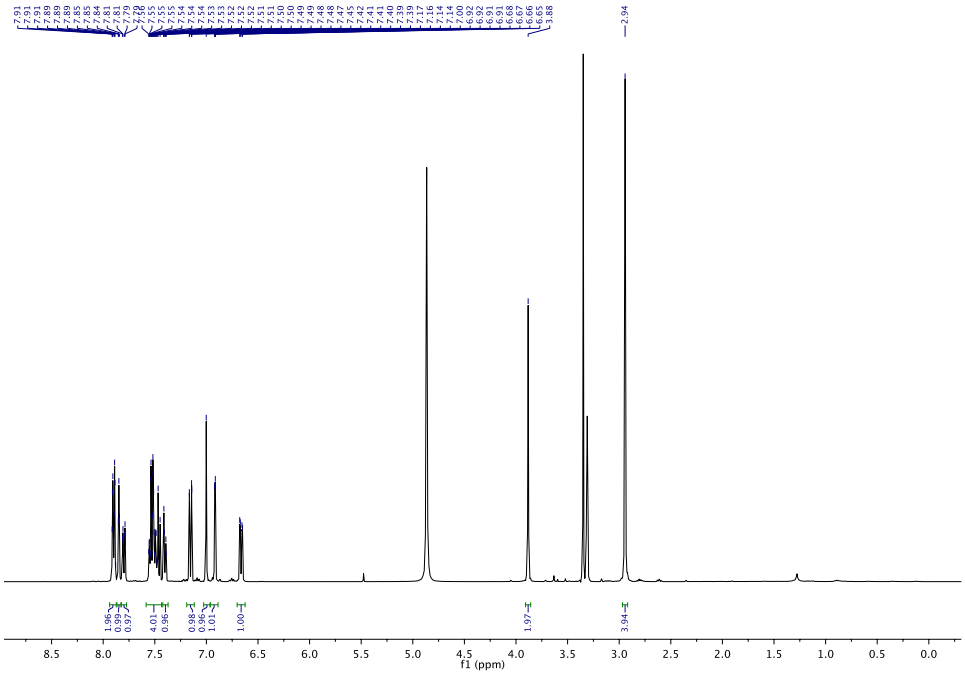


^{13}C NMR

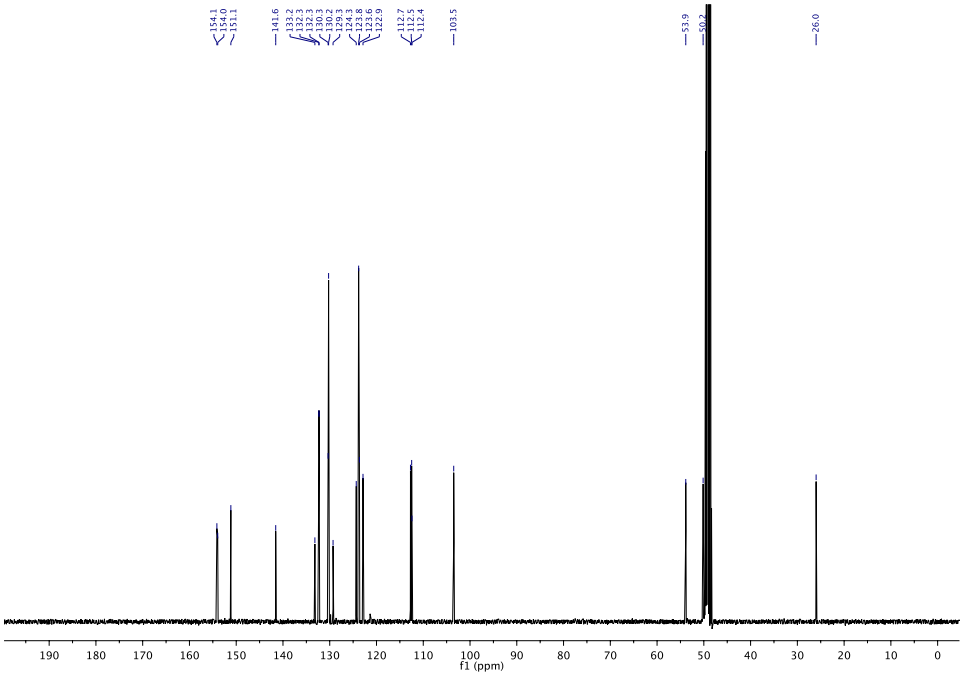


Azo5HT-2 (S2)

^{1}H NMR

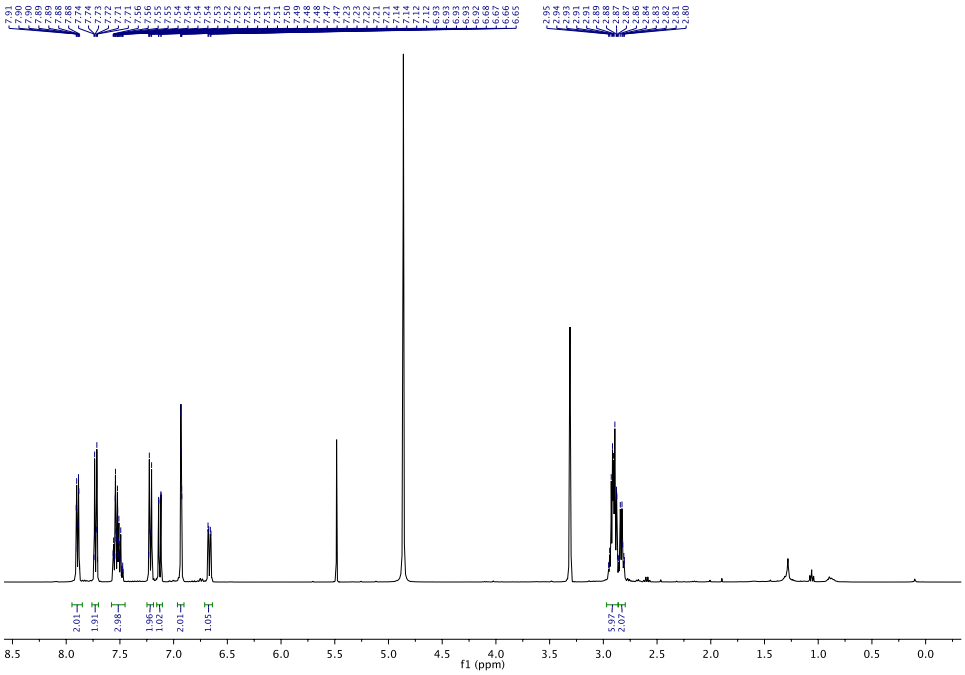


^{13}C NMR

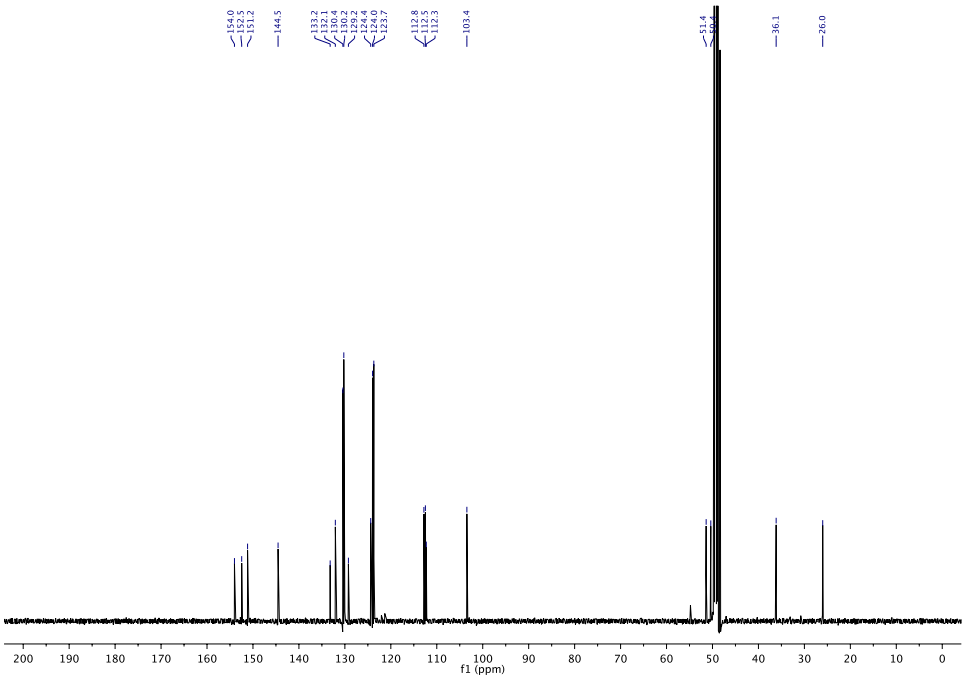


Azo5HT-3 (S3)

^{1}H NMR

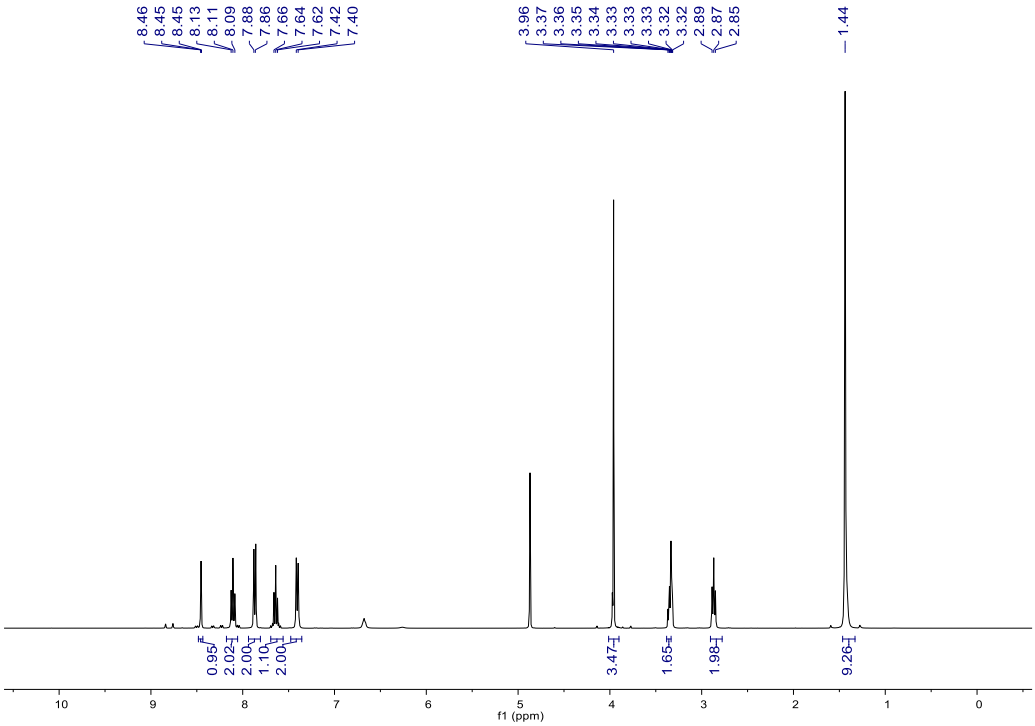


^{13}C NMR

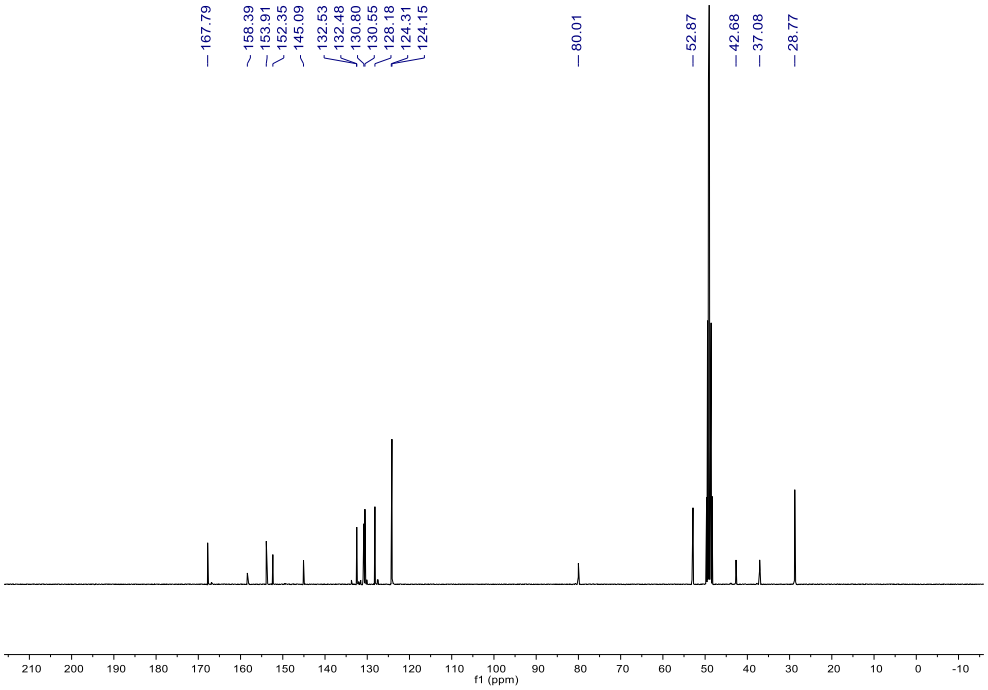


S4: methyl (E)-3-((4-(2-((tert-butoxycarbonyl)amino)ethyl)phenyl)diazenyl)benzoate

^{1}H NMR

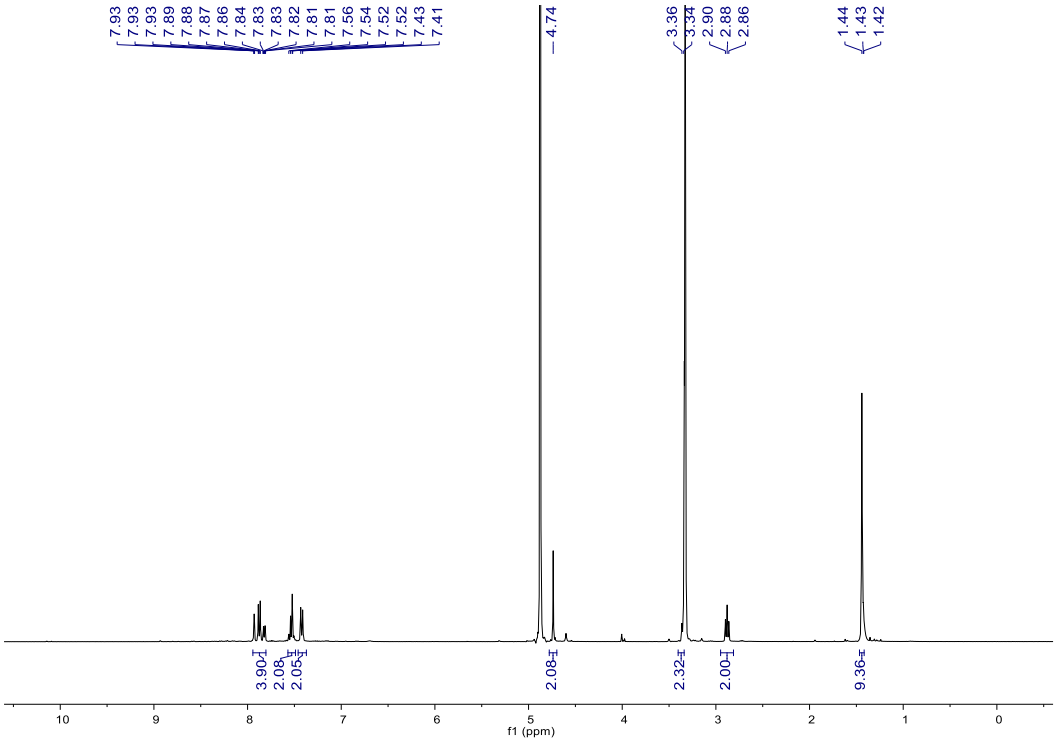


^{13}C NMR

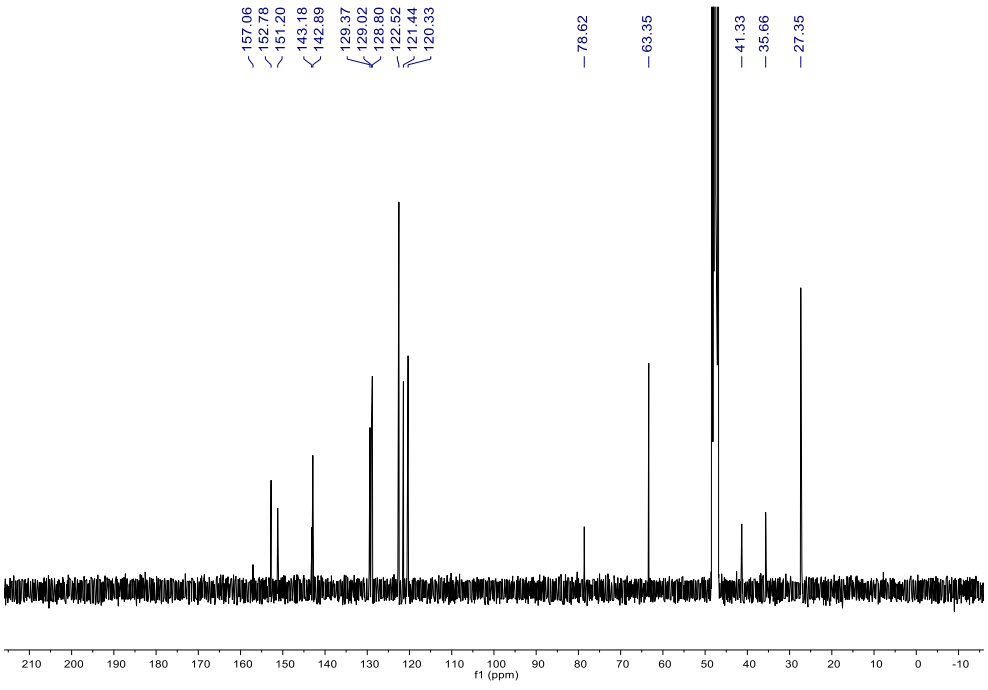


S5: tert-butyl (E)-(4-((3-(hydroxymethyl)phenyl)diazenyl)phenethyl)carbamate

^{1}H NMR

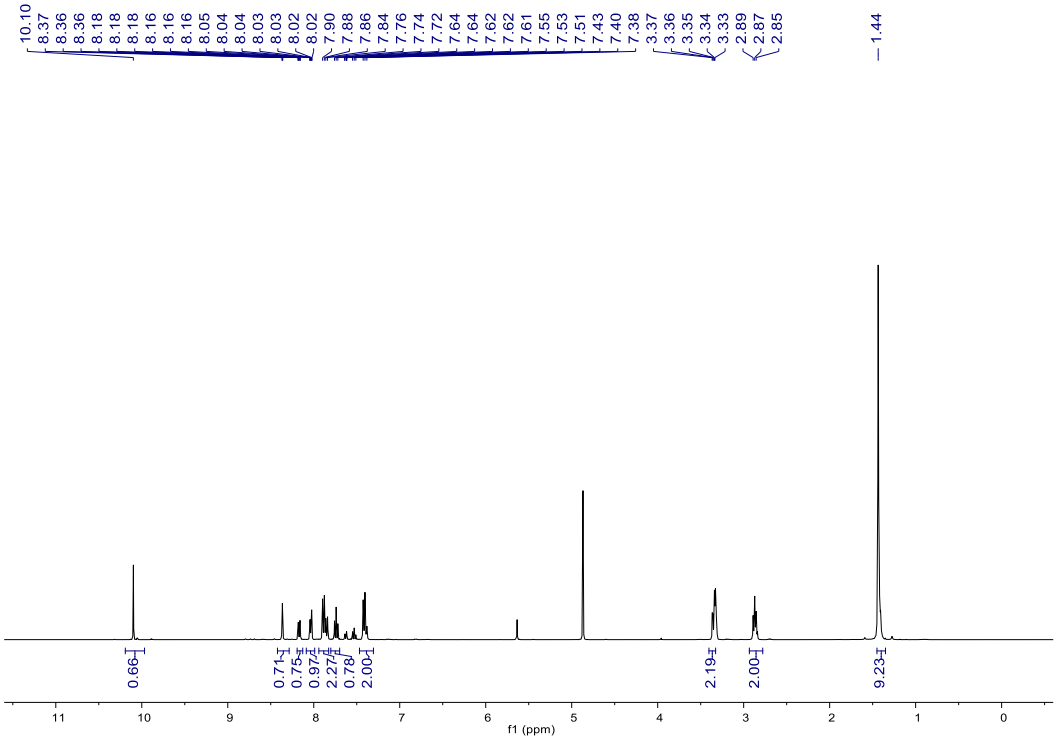


^{13}C NMR

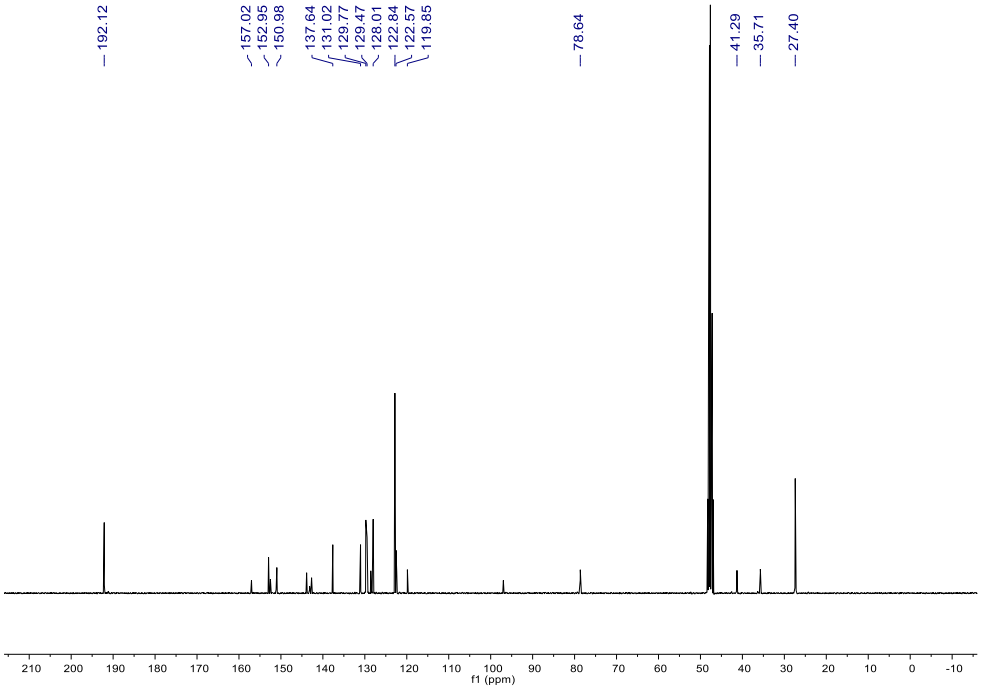


S6: tert-butyl (E)-(4-((3-formylphenyl)diazenyl)phenethyl)carbamate

^{1}H NMR

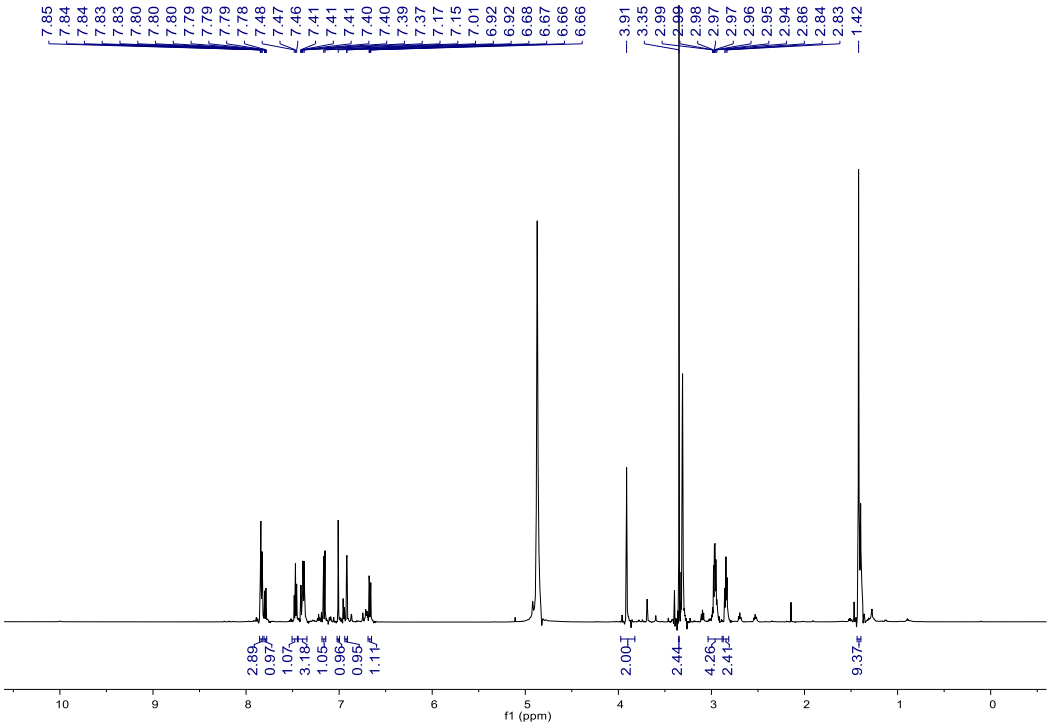


^{13}C NMR

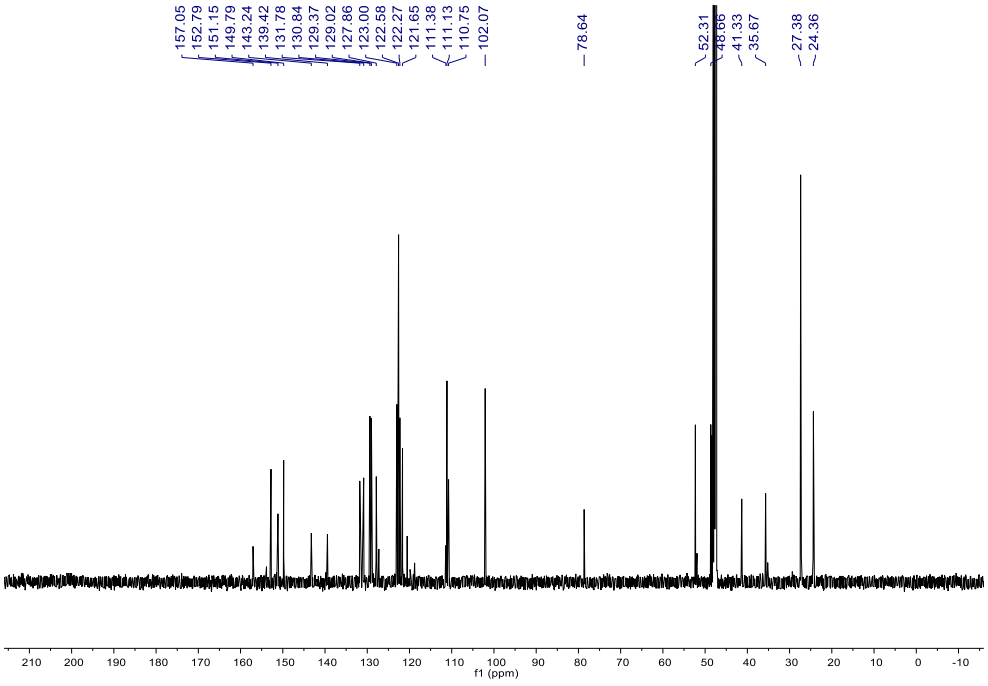


Boc-Azo5HT-2 (S7): tert-butyl (E)-(4-((3-(((2-(5-hydroxy-1H-indol-3-yl)ethyl)amino)methyl)phenyl)diazenyl)phenethyl)carbamate

^{1}H NMR

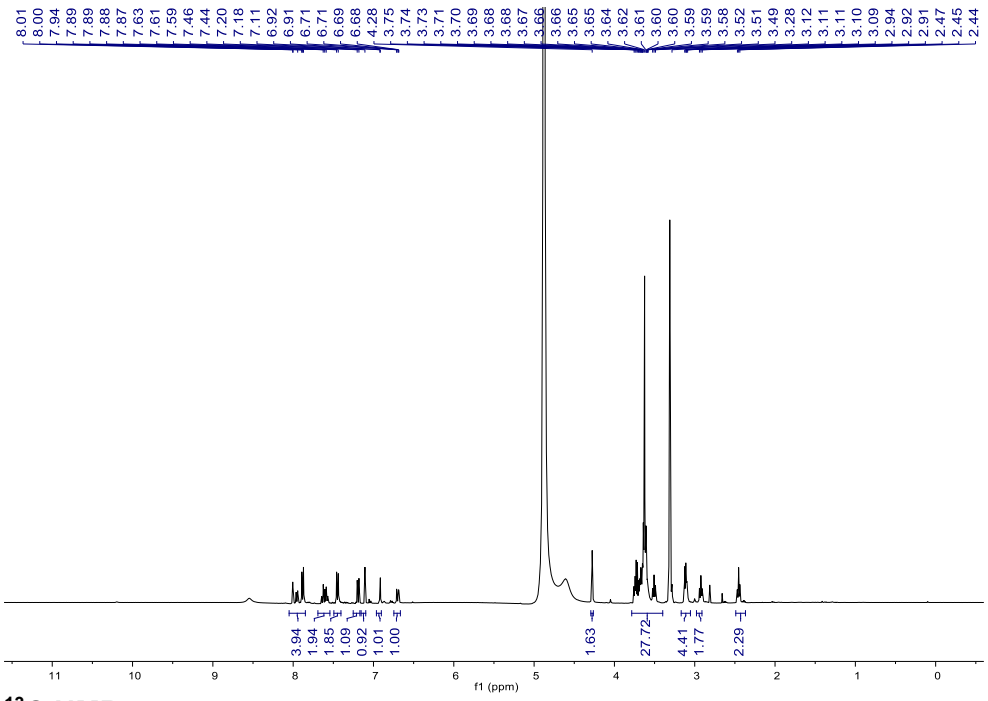


^{13}C NMR

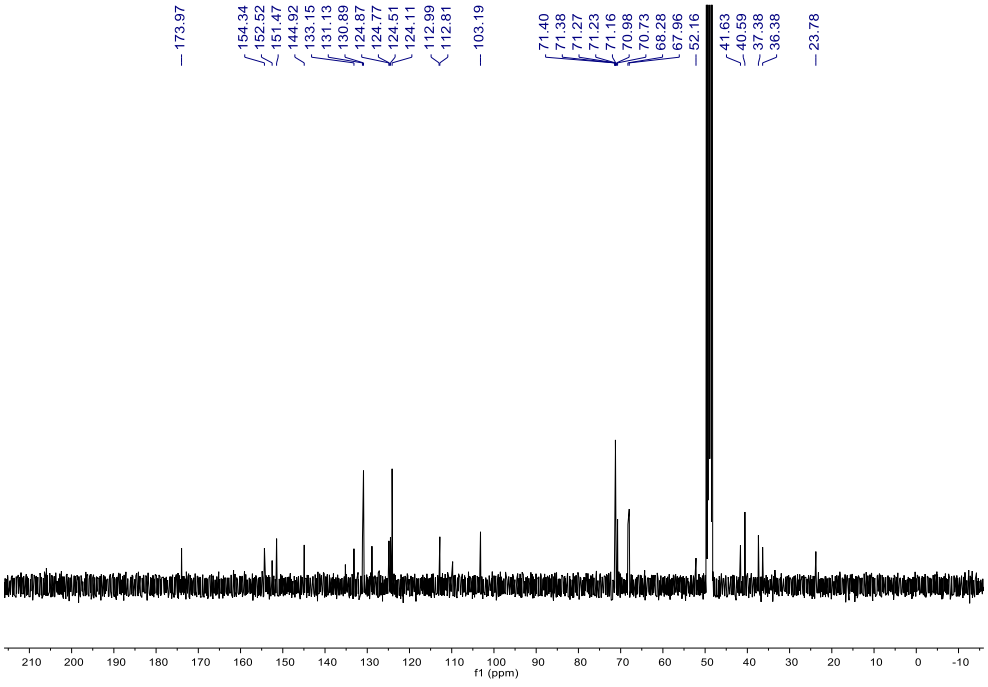


Azo5HT-PEG[6] (S8): (E)-1-amino-N-(4-((3-(((2-(5-hydroxy-1H-indol-3-yl)ethyl)amino)methyl)phenyl)diazenyl)phenethyl)-3,6,9,12,15,18-hexaoxahenicosan-21-amide

^{1}H NMR

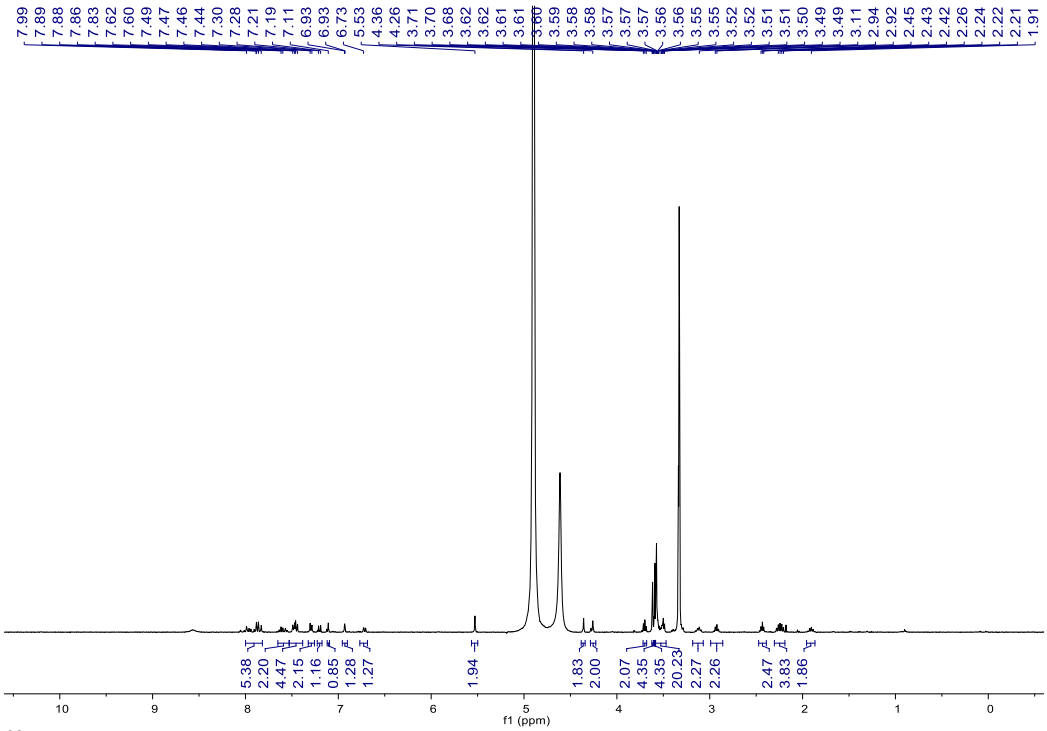


^{13}C NMR

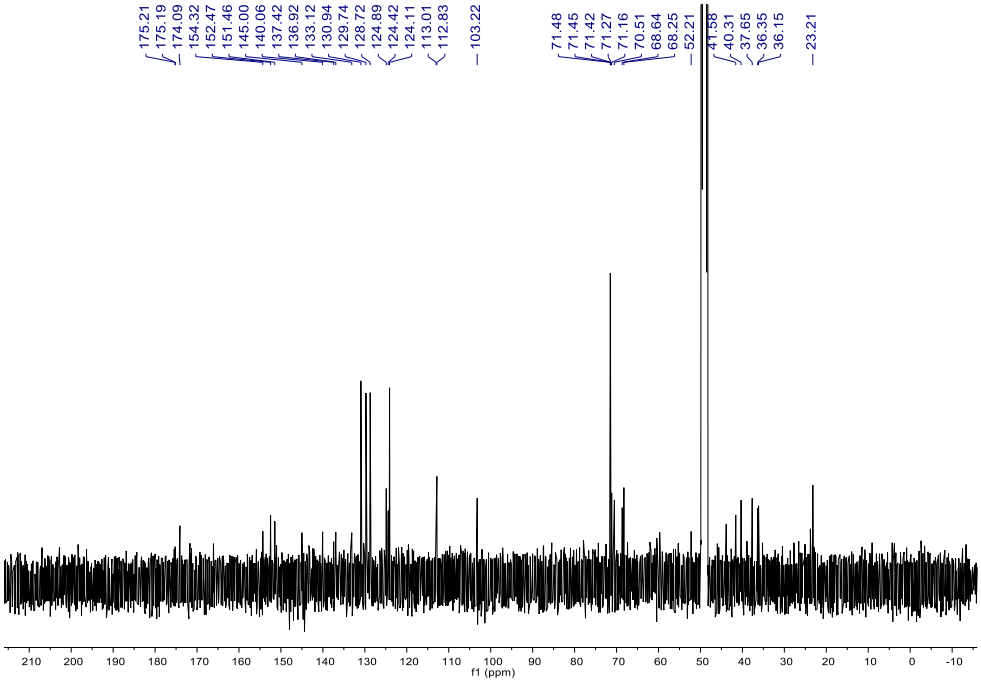


BGAzo5HT-PEG[6] (S9): (E)-N1-(4-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl)-N5-(24-(4-((3-(((2-(5-hydroxy-1H-indol-3-yl)ethyl)amino)methyl)phenyl)diazenyl)phenyl)-21-oxo-3,6,9,12,15,18-hexaoxa-22-azatetracosyl)glutaramide

^{1}H NMR

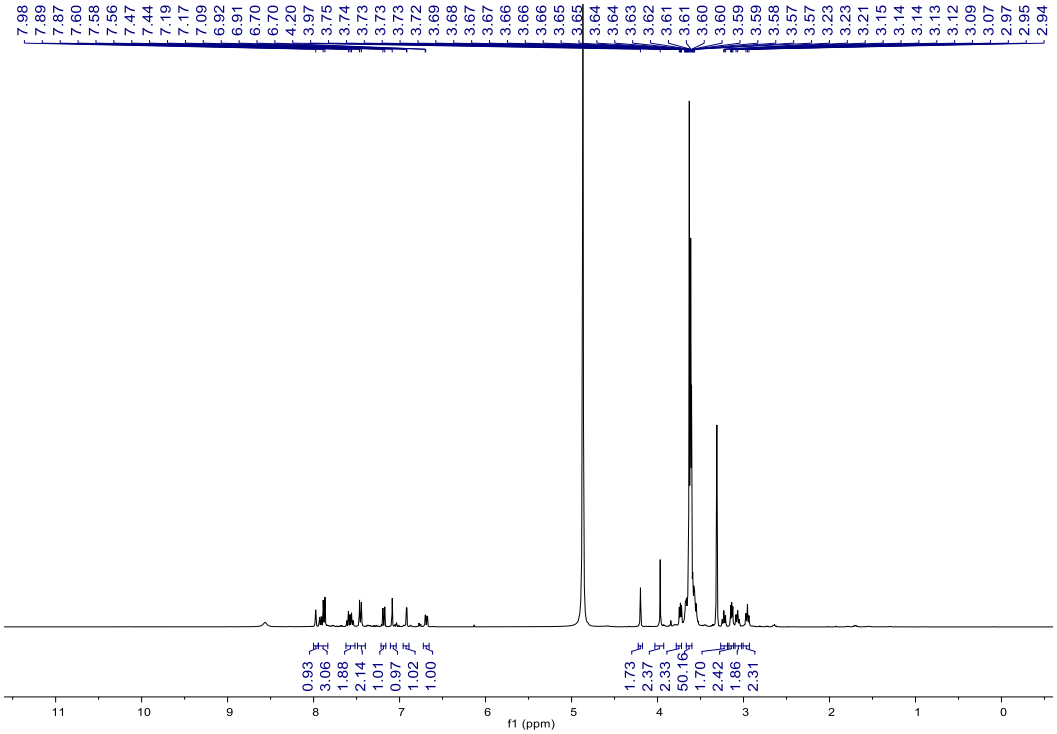


^{13}C NMR

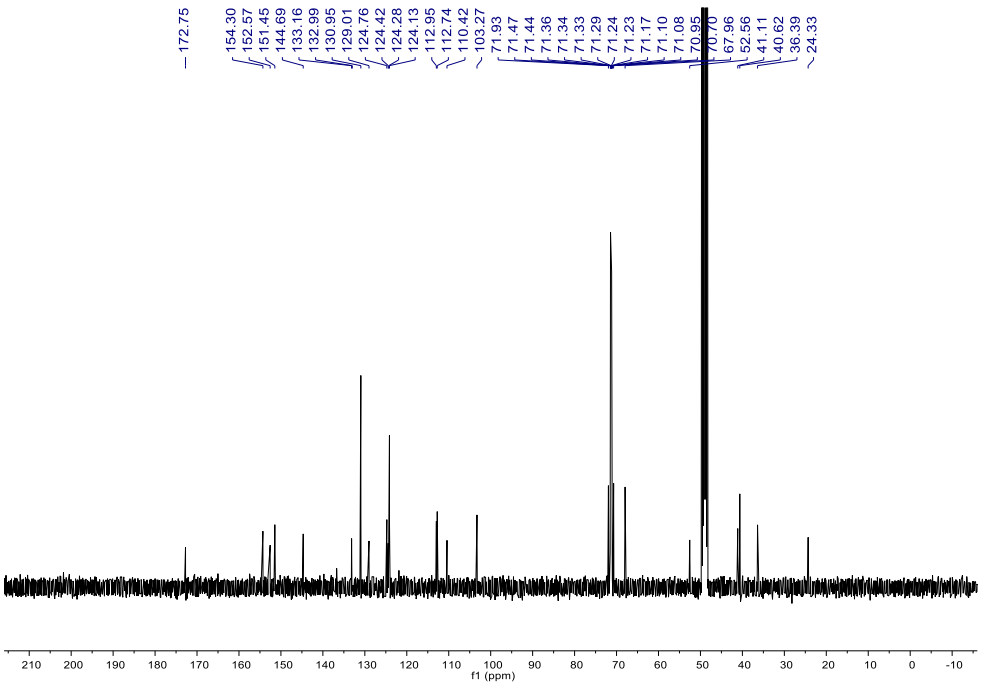


Azo5HT-PEG[12] (S10): (E)-38-amino-N-(4-((3-(((2-(5-hydroxy-1H-indol-3-yl)ethyl)amino)methyl)phenyl)diazenyl)phenethyl)-3,6,9,12,15,18,21,24,27,30,33,36-dodecaoxaoctatriacontanamide

^{1}H NMR

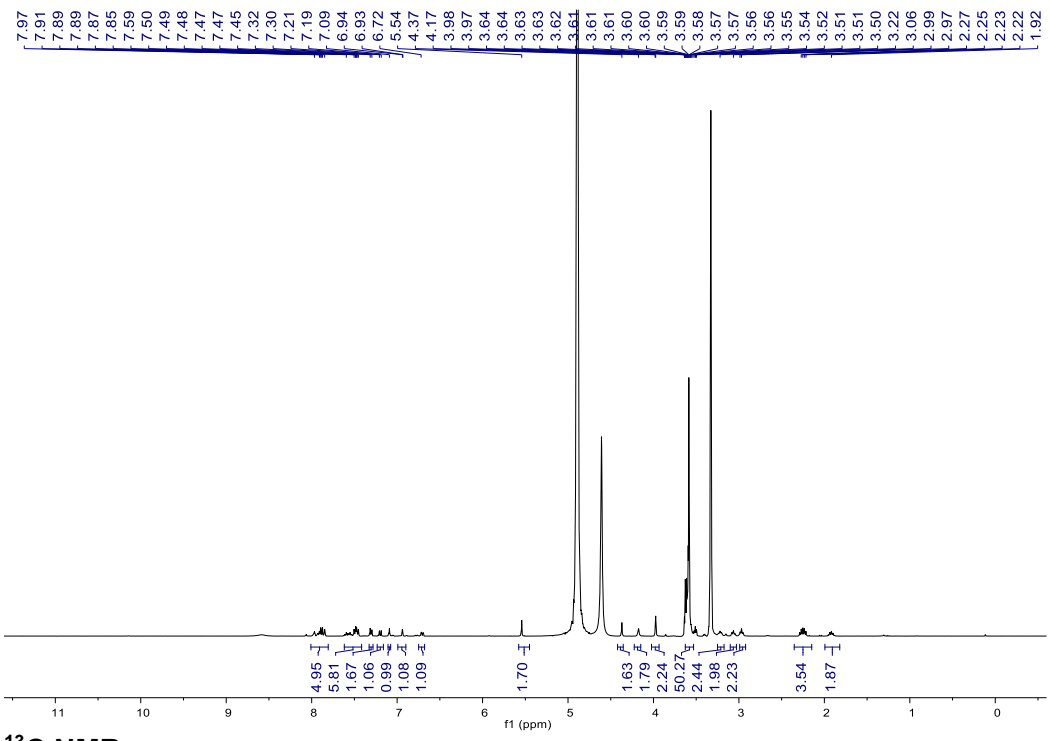


^{13}C NMR

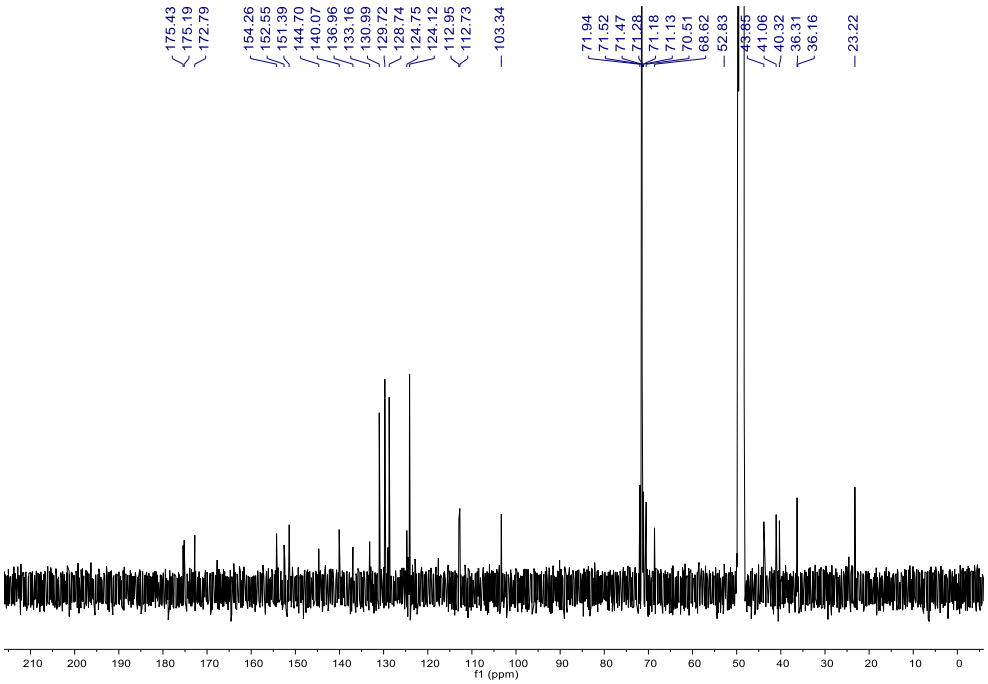


BGAzo5HT-PEG[12] (S11): (E)-N1-(4-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl)-N5-(1-(4-((3-(((2-(5-hydroxy-1H-indol-3-yl)ethyl)amino)methyl)phenyl)diazenyl)phenyl)-4-oxo-6,9,12,15,18,21,24,27,30,33,36,39-dodecaoxa-3-azahentetracontan-41-yl)glutaramide

^{1}H NMR

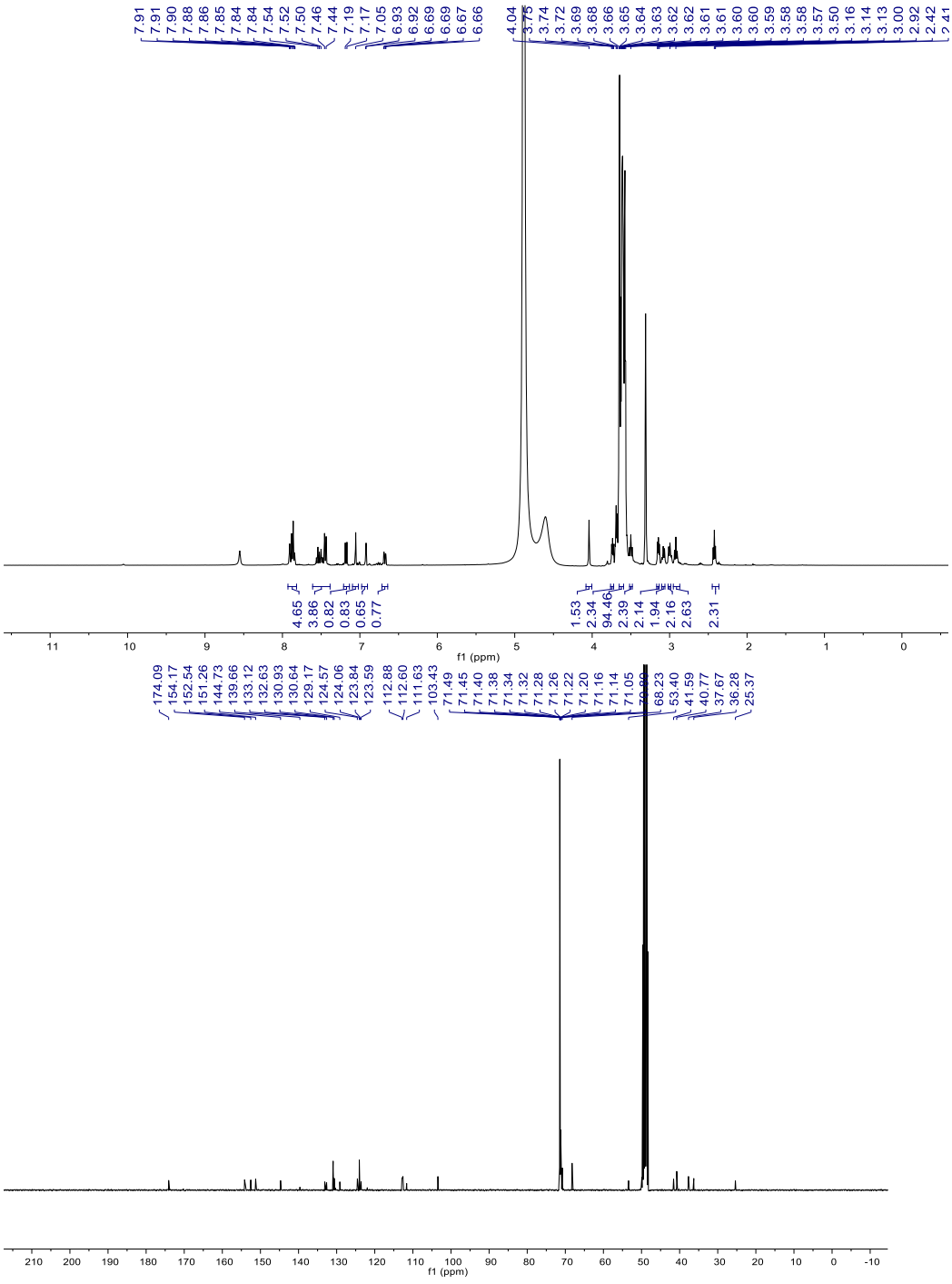


^{13}C NMR



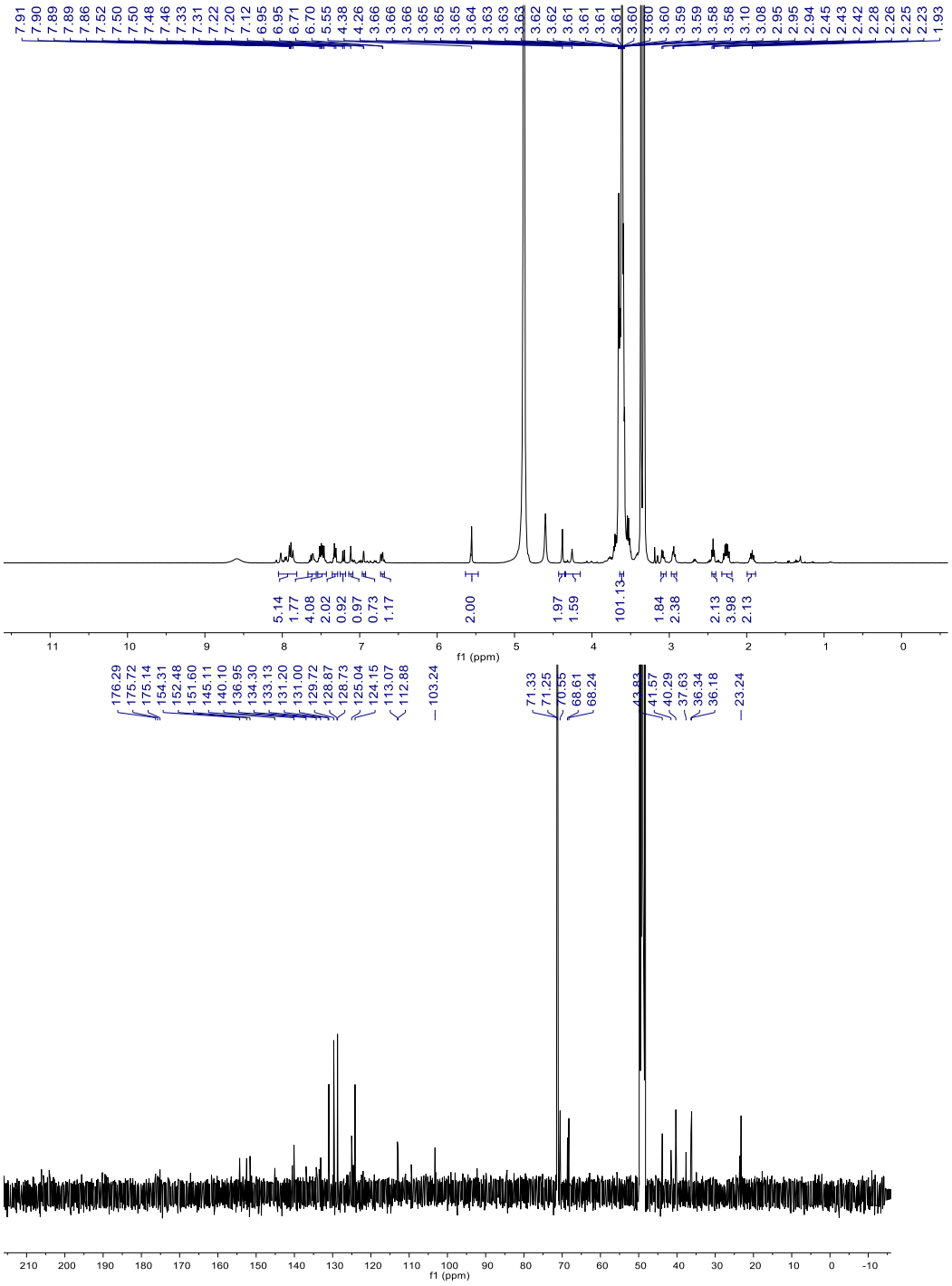
Azo5HT-PEG[24] (S12): (E)-1-amino-N-(4-((3-(((2-(5-hydroxy-1H-indol-3-yl)ethyl)amino)methyl)phenyl)diazenyl)phenethyl)-3,6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72-tetracosaoxapentaheptacontan-75-amide

^{1}H NMR



BGAzo5HT-PEG[24] (S13): (E)-N1-(4-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl)-N5-(78-(4-((3-(((2-(5-hydroxy-1H-indol-3-yl)ethyl)amino)methyl)phenyl)diazenyl)phenyl)-75-oxo-3,6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72-tetracosaoxa-76-azaoctaheptacontyl)glutaramide

^{1}H NMR



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