Supporting Information

Photoswitchable TCB-2 for Control of the $5HT_{2A}$ Receptor and Analysis of Biased Agonism

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General Experimental Procedures

Starting materials, reagents, and polymers were commercially available and used as obtained from Sigma-Aldrich, TCI, and Thermo-Fischer Scientific without further purification. TCB-2 was purchased from Tocris. Unless otherwise noted, all reactions were performed in oven-dried glassware under positive argon pressure. Thin layer chromatography (TLC) was performed on pre-coated silica gel plates (Sigma Aldrich) and visualized with UV light (λ = 254 nm). Flash-column chromatography was performed using silica gel (60 Å, 60-200 micrometer, Thermo-Fisher Scientific). NMR spectra were recorded with Bruker Avance spectrometers using deuterated solvents. ^{1}H NMR spectra were recorded at 400 MHz as indicated. ^{13}C spectra were recorded at 100 MHz. ^{1}H NMR data are reported in the following order: chemical shift (δ ppm), multiplicity, coupling constant (Hz), and integration. ^{13}C NMR data are reported in terms of chemical shift. UV-vis spectroscopy was performed in quartz cuvettes (10 mm path length) on UV-vis spectroscopy was performed on a Varian Cary Bio 50 UV-Vis spectrophotometer. Weighing small quantities was performed on a Mettler Toledo XS105 analytical microbalance. High-resolution mass spectra were obtained from the University of Delaware Mass Spectrometry Facility using an Orbitrap Q-Exactive instument.

Ca^{2+} Flux Assay

HEK293T cells was maintained in Dubeccos Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, in a humidified atmosphere at 37 °C in 5% CO2. On day 1, cells were plated at a density of 4x104 cells/cm2 in a poly-D-lysine coated 18-well chambered coverslip (Ibidi). The following day (day 2), cells were transfected with a 10x solution of 3:1 mixture of 5HT2A:Optifect Transfection Reagent (Thermo) in unsupplemented DMEM (this step was optimized with a fluorescent fusion protein). On day 3 the transfection media was removed and calcium sensitive dye loading was performed following the protocol of the manufacturer of Cal-590 AM (AAT). 1x drug stimulation solutions were prepared in filter-sterilized HBSS. Once Cal-590 loading was complete, a time series acquisition at a rate of one frame/second was recorded using a Zeiss LSM 980 with Airyscan 2. Basal fluorescence was recorded for 20 seconds, followed by addition of drug solution to a 1x final concentration and acquisition for an additional 40 seconds. Results in the form of fold fluorescence increase over basal were averaged over at least 20 cells in Zeiss Zen software and GraphPad Prism was used for analysis of data, normalizing to the initial baseline.

PRESTO-TANGO Beta Arrestin Assay[1]

HTLA cells were a gift from the laboratory of G. Barnea and were maintained in Dubeccos Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 2 µg/ml puromycin, 100 µg/ml hygromycin B, and 100 µg/ml G418, in a humidified atmosphere at 37 °C in 5% CO_2. On day 1, cells were plated at a density of 1x105 cells/cm^2 in a black wall, clear bottom 96 well plate (Nunc). The following day (day 2), cells were transfected with a 10x solution of 3:1 mixture of 5HT2A-TANGO:Optifect Transfection Reagent (Thermo) in un-supplemented DMEM. On day 3, 1× drug stimulation solutions were prepared in filter-sterilized unsupplemented DMEM. The transfection media was shaken or aspirated from the wells, and drug stimulation

solutions were gently added. After 4 h, the drug solutions were aspirated and media was replaced. On day 4, media was removed from one well every ten seconds (to maintain consistency of incubation time) and 50 µl per well of Bright-Glo solution (Promega) diluted 20-fold in HBSS was added. After incubation for 2 min at room temperature, luminescence was counted with an integration time of 10 sec in a Spectramax i3x plate reader (Molecular Devices).

Photo-Irradiation

Irradiation at 365 nm was performed with a UV transilluminator (Chem Glass).
Irradiation at 530 was done with an LED (Thorlabs M530L4) with a collimation adaptor (SM2F32-A) at the focal point.

Determination of Photostationary State (PSS) by ^{1}H NMR Spectroscopy

A stock solution of each photoswitch was prepared in d_6DMSO, and the initial spectrum (dark thermal distribution of isomers) is recorded. To determine $PSS_{E->Z}$ the sample is irradiated at 365 nm in 30 second intervals and NMR immediately recorded. To determine the $PSS_{Z->E}$ the sample is first irradiated to PSS with 365 nm light, then irradiated with 530 nm light at 30 second intervals and NMR recorded until a stationary isomer distribution is reached. Analysis of the relative integrations of the isomer peaks was used to determine the distribution of isomers at PSS.

Fig. S1 PSS Determination for Azo-Ald. Both states were reached after 5 min irradiation.

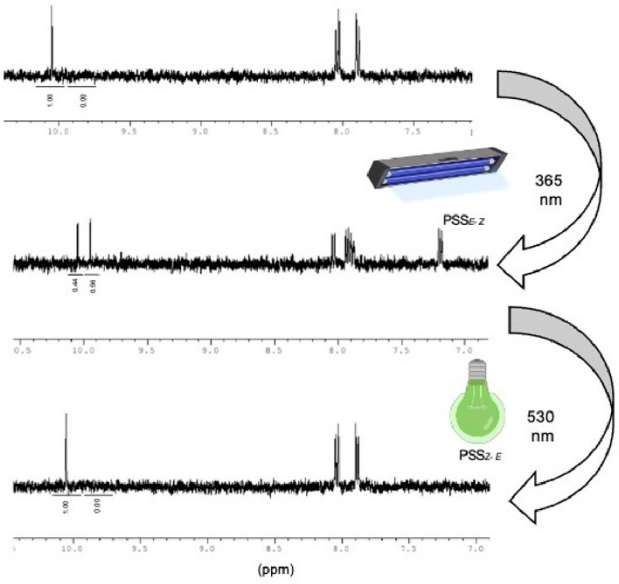
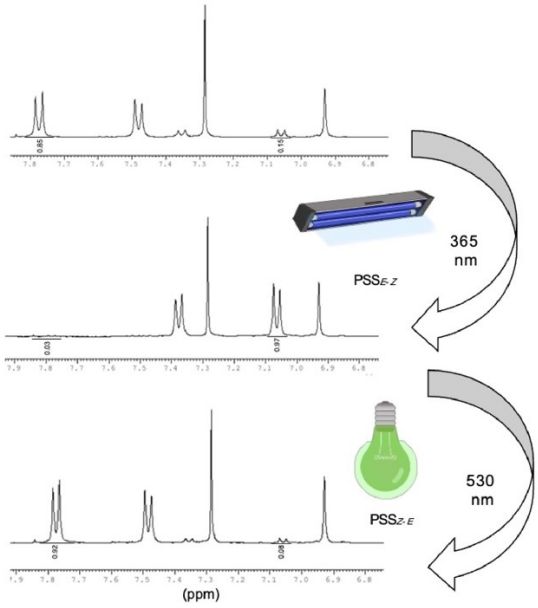


Fig. S2 PSS Determination for Azo-TCB2. Both states were reached after 5 min irradiation.



Determination of Thermal *Z*→*E* isomerization

Thermal Isomerization Calculations

The thermally induced spontaneous isomerization of the *Z* isomer to the stable *E* isomer is assumed to follow first order kinetics which may be modeled as a one-phase exponential decay function.[2] The curve fitting was performed using the GraphPad program.

Fig. S3 Thermal *Z*→E isomerization of Azo Ald**, monitored by ^{1}H NMR, 20 μM concentration used due to the high concentration-dependence of irradiation of this molecule.

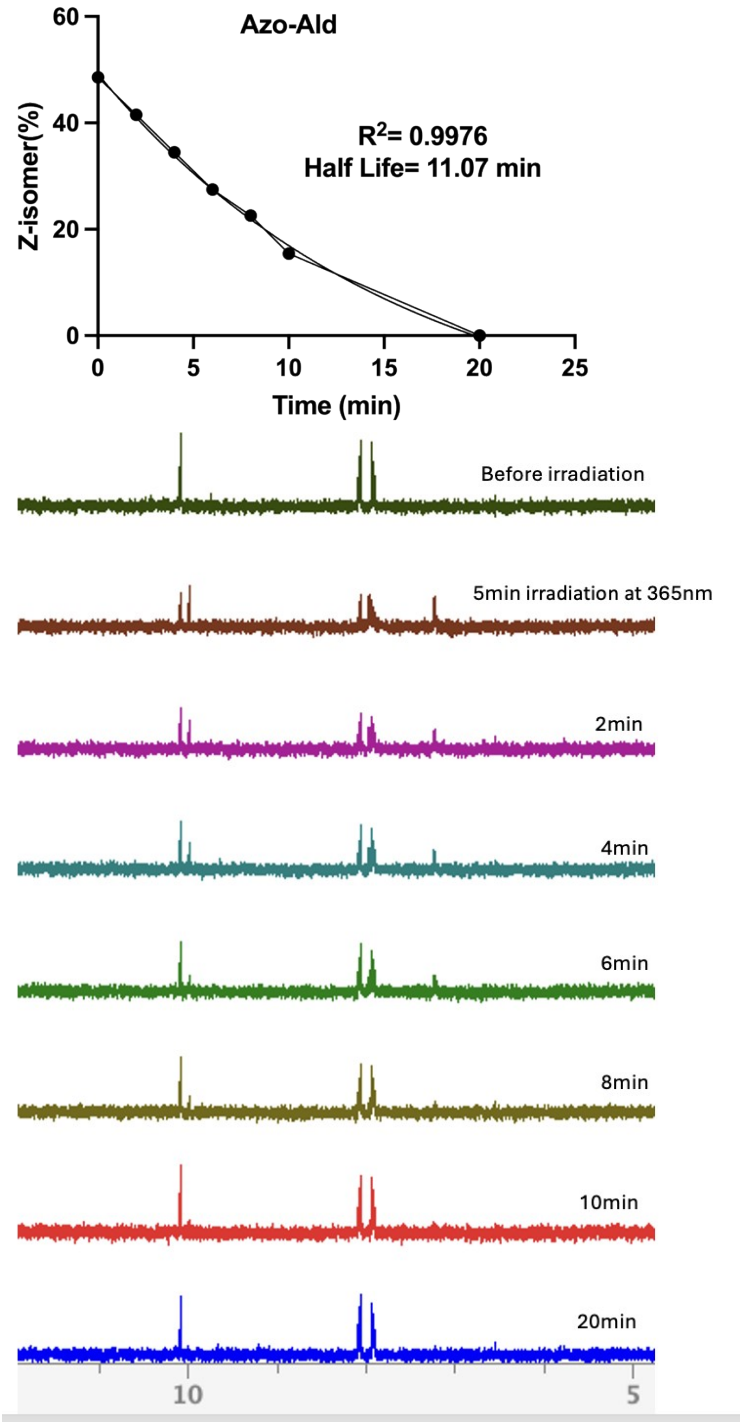
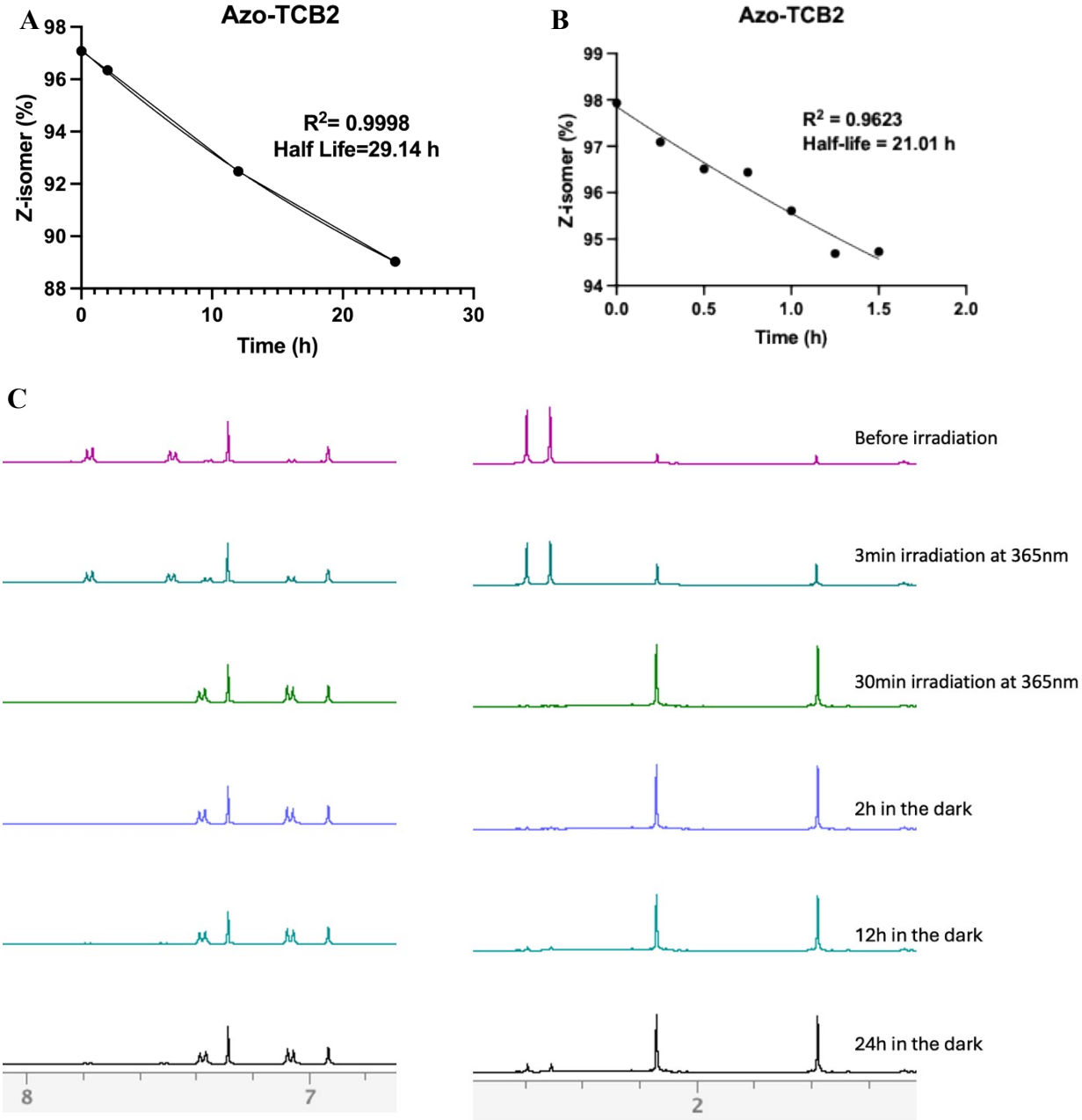


Fig. S4 Thermal *Z*→E isomerization of Azo-TCB2, monitored by ^{1}H NMR, 10 mM concentration. **A**: 25 ºC; **B**: 37 ºC; **C**: example NMR data for 25 ºC.

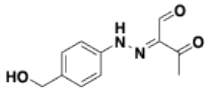


Determination of Quantum Yield

Quantum yield was determined according to the protocol of Jeong *et al*.[3] To determine molar photon flux at the cuvette, a ferrioxolate actinometer was used, with ferrioxolate concentrations at 0.006 M for 365 nM and 0.15 M at 530 nM due to the poor absorbance at this wavelength.[3–5]

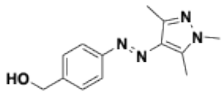
Synthetic Procedures

3-(2-(4-(Hydroxymethyl)phenyl)hydrazineylidene)pentane-2,4-dione (1).[6]



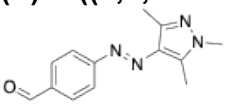
Synthesized following the method of Courtney *et al.*, spectra matched those reported.[6]

(*E*)-(4-((1,3,5-Trimethyl-1H-pyrazol-4-yl)diazenyl)phenyl)methanol (2).[6]



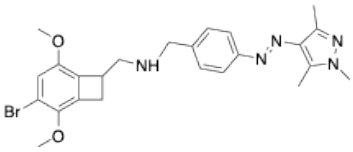
Synthesized according to the method of Courtney *et al.* with modifications to accommodate the limited commercial availability of methylhydrazine, which was replaced with the readily commercially available reagent methylhydrazine sulfate.[6] Diketone **1** (0.5 g, 2.1 mmol, 1.0 equiv.) was dissolved in ethanol (20 mL) and heated to reflux. A mixture of methylhydrazine sulfate (4.54 g, 31.5 mmol, 15.0 equiv.) and potassium carbonate (4.35 g, 31.5 mmol, 15.0 equiv.) in 5 mL ethanol was added, and the combined solution heated to reflux for 2.5 h. The mixture is then concentrated in vacuo and purified via Biotage™ silica gel flash chromatography (MeOH in CH_2Cl_2 0→10%) to provide the title compound (0.257 g, 1.05 mmol, 50%), spectra matched those reported.[6]

(*E*)-4-((1,3,5-trimethyl-1H-pyrazol-4-yl)diazenyl)benzaldehyde (Azo-Ald).



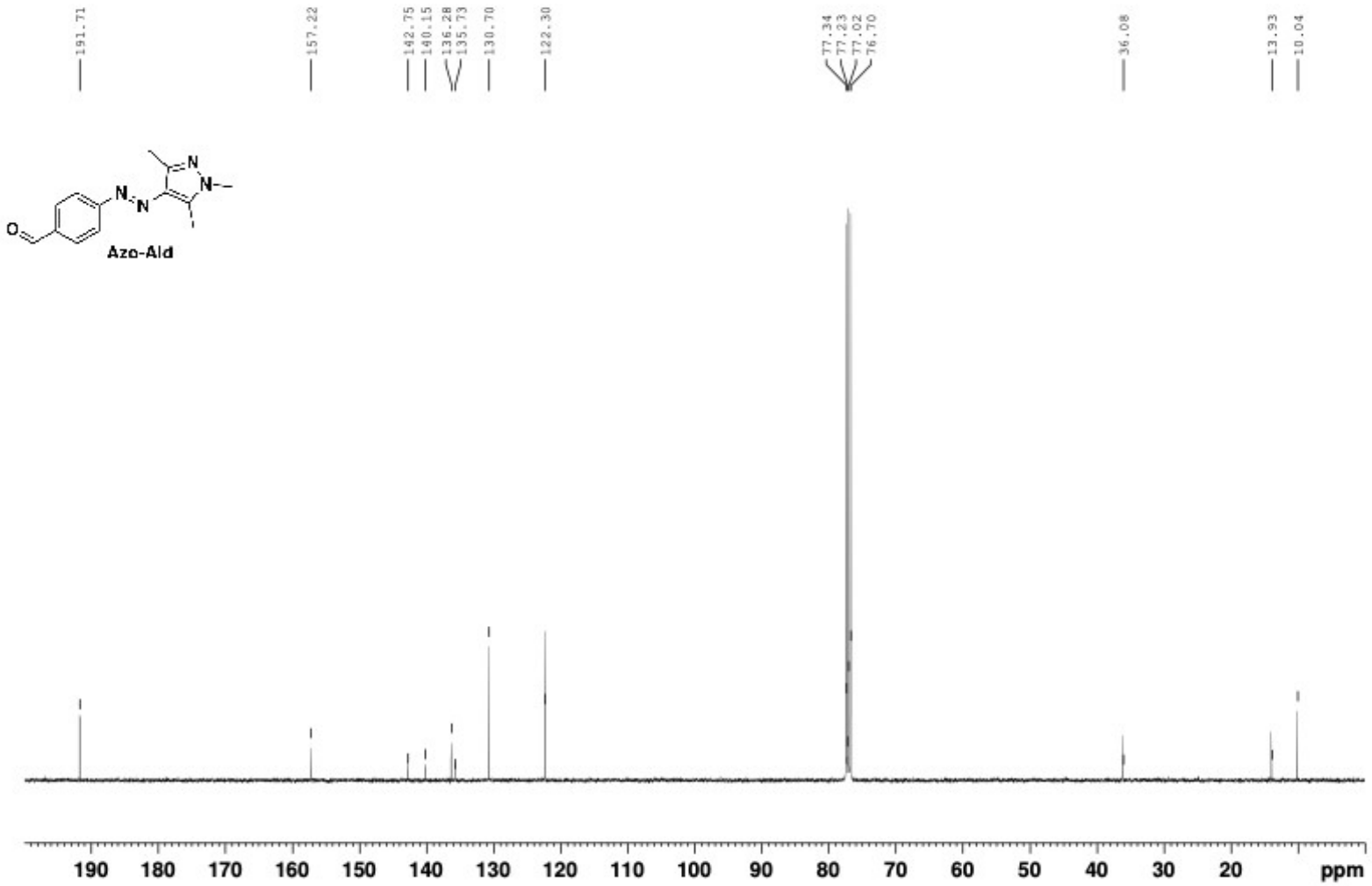
To a solution of alcohol **2** (0.244 g, 1.0 mmol, 1.0 equiv.) in $CHCl_3$ (25 mL) is added MnO_2 (O.435 g, 5.0 mmol, 5 equiv.) and the mixture stirred at room temperature for 12 h. The mixture is then filtered over Celite® and concentrated via rotary evaporator and purified via Biotage™ silica gel flash chromatography (MeOH in CH_2Cl_2 0→10%) to provide the title compound (0.189 g, 0.78 mmol, 78%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.06 (s, 1H), 7.96 (d, *J*=8 Hz, 2H), 7.90 (d, *J*=8 Hz, 2H) 3.80 (s, 3H), 2.60 (s, 3H), 2.51 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.7, 157.2, 142.8, 140.2, 136.3, 135.7, 130.7, 122.3, 36.1, 13.9, 10.0; HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{13}H_{15}ON_4$ 243.1240; found 243.1235.

(*E*)-1-(3-bromo-2,5-dimethoxybicyclo[4.2.0]octa-1,3,5-trien-7-yl)-N-(4-((1,3,5-trimethyl-1H-pyrazol-4-yl)diazenyl)benzyl)methanamine (Azo-TCB2). To TCB-2 hydrobromide



(20.0 mg, 0.057 mmol, 1.0 equiv.), and azo aldehyde (13.7 mg, 0.057 mol, 1.0 equiv.) were dissolved in 1.0 mL ethanol, triethylamine (12 mg, 0.016 mL, 0.11 mmol, 2.0 equiv.), and the solution allowed to stir at room temperature for 20 min. Then sodium cyanoborohydride (7.2 mg, 0.11 mmol mol, 2.0 equiv.) was then added, and the mixture allowed to stir overnight. Ethanol was removed with reduced pressure and the mixture was purified by reversed phase biotage (ACN in H_2O, 0→100%) and lyophilized to provide **Azo-TCB2** (0.045 mmol, 22.3 mg, 79%) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J*=8.4 Hz, 2H), 7.47 (d, *J*=8.4 Hz, 2H), 6.91 (s, 1H), 4.03 (m, 5H), 3.81 (m, 7H), 3.55 (dd, *J*=13.5, 5.2 Hz, 1H), 3.07 (m, 3H), 2.58 (S, 3H), 2.50 (S, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.0, 148.3, 145.6, 142.4, 138.8, 135.1, 132.8, 128.9, 127.7, 121.9, 115.8, 109.8, 57.8, 56.0, 53.2, 52.4, 41.5, 36.0, 34.6, 13.8, 10.0; HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{24}H_{29}BrN_5O_2$ 498.1499, found 498.1478.

10.057
7.964
7.901
3.803
2.603
2.509
2.166
2.040
1.252
Azo-Ald
0.99
2.08
2.05
3.00
2.98
3.00
ppm


191.71
157.22
142.75
140.15
136.28
135.73
130.70
122.30
77.34
77.23
77.02
76.70
36.08
13.93
10.04
Azo-Ald
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170
160
150
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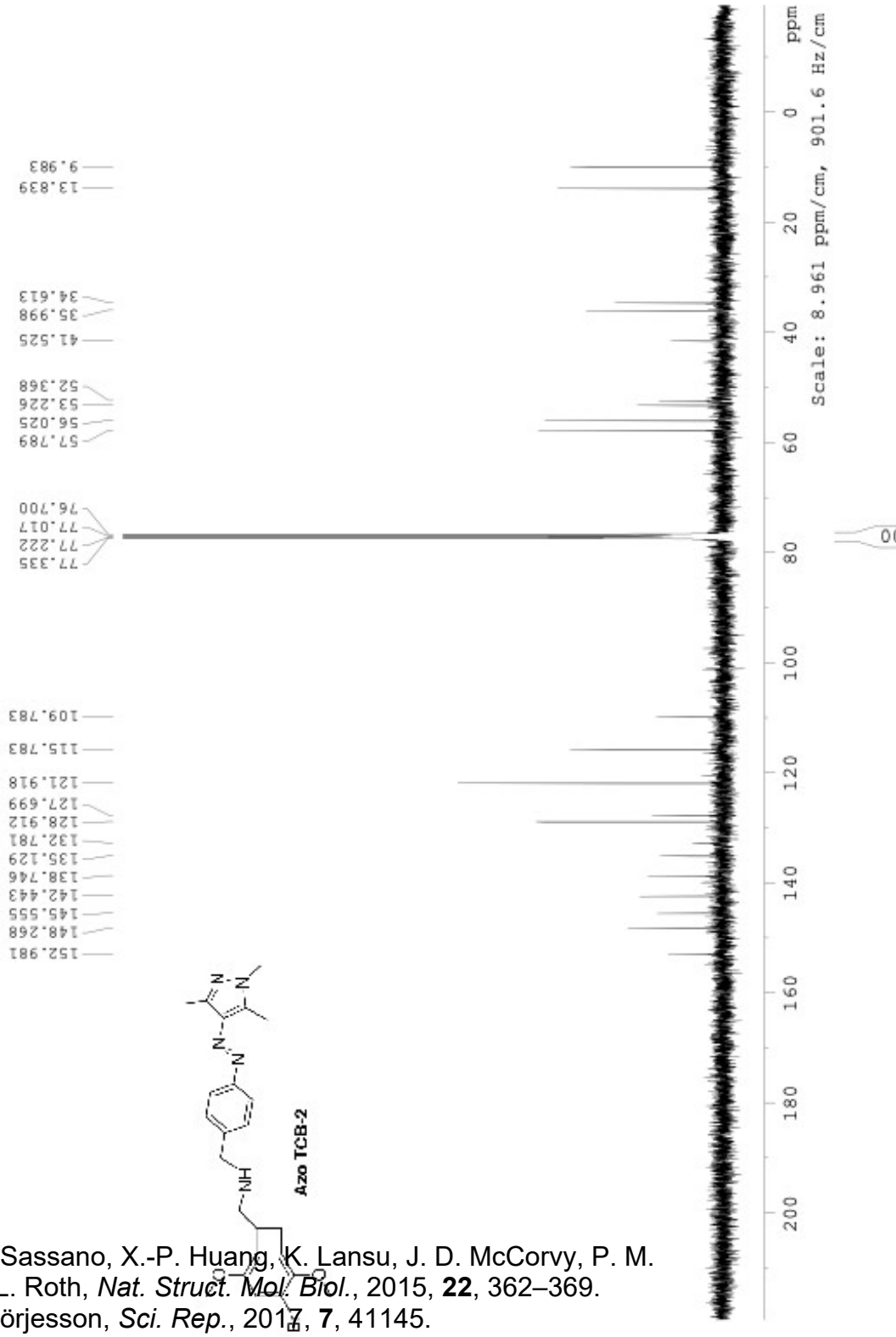
130
120
110
100
90
80
70
60
50
40
30
20
ppm



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