Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supplementary Information

Azogabazine: a photochromic antagonist for the $GABA_A$ receptor

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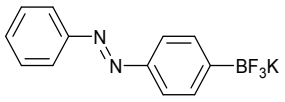
Contents

General experimental

All reactions were carried out at atmospheric pressure with stirring unless otherwise stated. All reagents and solvents were purchased from suppliers and used without further purification unless otherwise stated. All reactions were monitored by TLC or ^{1}H NMR as stated. TLC plates pre-coated with silica gel 60 F254 on aluminium (Merck KGaA) were used, being visualized by UV (254 or 365 nm) or chemical stain ($KMnO_4$, I_2, ninhydrin). Normal phase silica gel (BDH) was used for flash chromatography and the eluting solvent or solvent gradient given. ^{1}H NMR and were recorded at 600 MHz and ^{13}C NMR spectra were recorded at 150 MHz on a Bruker AMX600-cryoprobe respectively, at ambient temperature, unless otherwise stated; all chemical shifts are measure in ppm and referenced to the residual proton impurity of the deuterated solvent. The multiplicity of the signal is indicated as s (singlet), d (doublet), t (triplet), dd (doublet of doublets), dt (doublet of triplets), quint (quintet) or m (multiplet), which is defined as all signals where overlap or complex coupling makes definitive descriptions of peaks difficult. All peaks should be taken as sharp unless otherwise described. Coupling constants are defined as *J* given in Hz. For NMR experiments, $CDCl_3$ denotes deuterated (d_3) chloroform, DMSO denotes deuterated (d_6) dimethylsulfoxide, and CD_3OD denotes deuterated (d_4) methanol. Deuterated solvents were chosen according to the position of solvent peak in spectra and solubility of substrate. High and low resolution mass spectrometry was performed using a VG70 SE operating in modes CI, EI, ES and FAB. Infrared spectra were obtained on a Perkin Elmer Spectrum 100 ATR FTIR Spectrometer or a Bruker Alpha Platinum ATR FT-IR spectrometer. Melting points were measured with a Gallenkamp apparatus and given with recrystallisation solvent where appropriate. Room temperature (rt) is defined as between 19-22 ºC. *In vacuo* is used to describe solvent removal by rotaryevaporation between 20 ºC and 60 ºC, at approximately 10 mmHg unless otherwise stated. The term "dried" refers to the process of adding then filtering away solid magnesium sulfate from an organic solvent to remove trace amounts of water. The term 'degassed' refers to the process of removing oxygen from a solution by bubbling argon through the solution prior to use. Microwave irradiation was carried out in a CEM 150W microwave reactor.

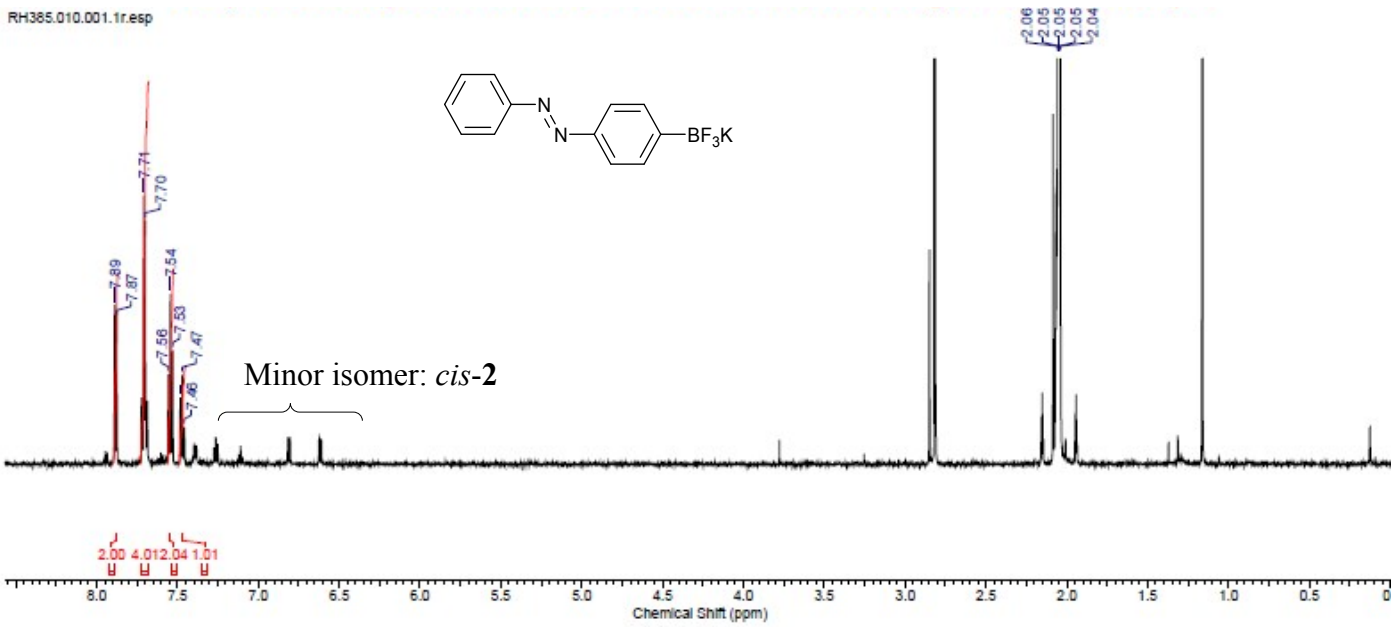
(*E*)-1-Phenyl-2-(4-(trifluoro-l4-boranyl)phenyl)diazene, potassium salt (2).[1]



To a stirring suspension of nitrosobenzene (489 mg, 4.56 mmol) in glacial acetic acid (45 mL) was added 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline (1.00 g, 4.56 mmol) and the reaction mixture was stirred at 90 °C for 3.5 h. The reaction solvent was removed *in vacuo* until 10 mL remained. The resulting mixture was diluted with H_2O (150 mL) and extracted with CH_2Cl_2 (3×150 mL). The combined organic layers were washed sequentially with H_2O (150 mL), saturated aqueous $NaHCO_3$ (150 mL), then H_2O (150 mL) and dried ($MgSO_4$) and concentrated *in vacuo*. The crude mixture was suspended in 40-60 °C petrol (100 mL) then filtered. To this crude precipitate were added CH_3CN (3 mL), H_2O (0.3 mL), and KHF_2 (1.07 g, 13.7 mmol). The resulting solution was stirred at rt for 2.5 h. After this the reaction mixture was quenched with saturated aqueous $NaHCO_3$ (0.5 mL) and the solvent removed *in vacuo*. The residue was cooled to 0 °C and 40-60 °C petrol (25 mL) was added. The resulting suspension was then filtered through a short column of Celite®, and the precipitate at the top of the column was washed with petrol (25 mL) and the filtrate discarded. The precipitate was then dissolved in acetone (100 mL) and filtered through the column. This acetone filtrate was then concentrated *in vacuo* to give trifluoroborate salt **2** (910 mg, 69%) as a brown solid.

Product exists as ratio *trans*-**2** : *cis*-**2** of 1.00:0.18 under ambient light.

mp 284-285 °C (from acetone); ν_{max}/ cm^{-1} 3643, 3040; δ_H (600 MHz, acetone-d6, Me_4Si) [*trans*- isomer data listed only] 7.50 (t, J = 7.5, 1H), 7.57 (t, J = 7.5, 2H), 7.70 (d, J = 8.3, 2H), 7.74 (d, J = 8.3, 2H), 7.90 (m, 2H); δ_C (150 MHz, acetone-d6, Me_4Si) 120.9 (CH), 122.3 (CH), 129.1 (CH), 130.3 (CH), 132.3 (CH), 150.9 (C), 152.9 (C) {1 C signal unobserved}; m/z (ES^-) 246 (M^-, 100), 248 (50).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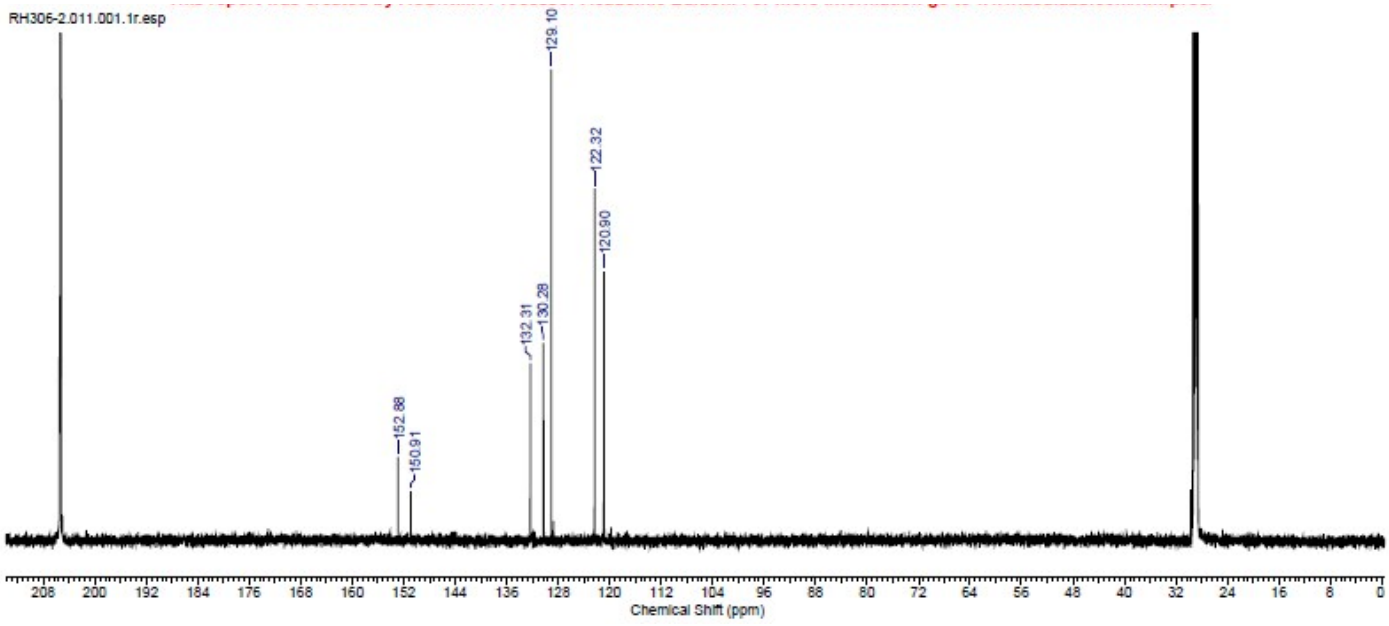
Minor isomer: *cis*-**2**
Chemical Shift (ppm)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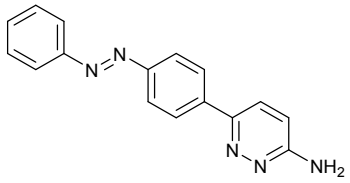
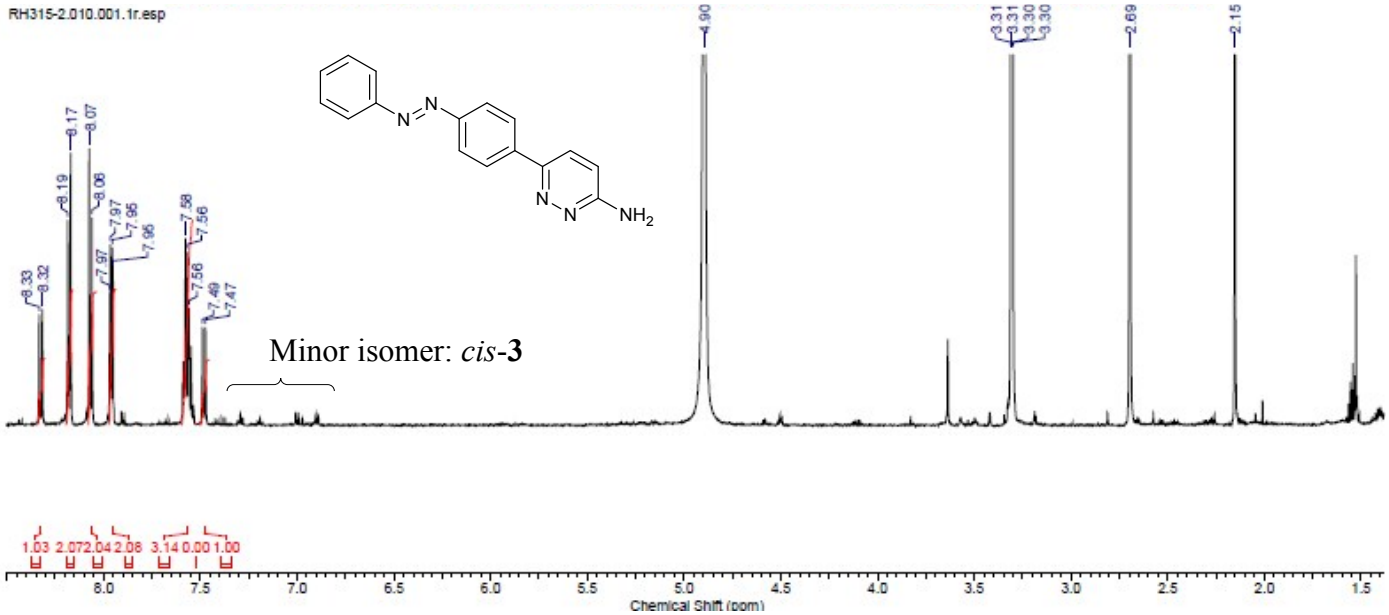
Chemical Shift (ppm)

6-(4-(Phenyldiazenyl)phenyl)pyridazin-3-amine, 3.



A microwave vial containing CH_3CN (2.0 mL) and H_2O (1.3 mL) was charged with trifluoroborate **2** (75 mg, 0.26 mmol), 3-amino-6-chloropyridazine (30.6 mg, 0.237 mmol), $Pd(PPh_3)_2Cl_2$ (8.3 mg, 0.012 mmol) and K_2CO_3 (98.3 mg, 0.711 mmol) and the reaction was degassed for 5 min, then microwaved for 15 min at 120 °C. The crude reaction mixture was concentrated *in vacuo*. Purification *via* flash column chromatography (40-60 °C petrol : EtOAc, 2:1 to 1:3) gave **3** (53.8 mg, 41%) as an orange solid.

mp 222-224 °C (from EtOH); ν_{max} (oil) 3351, 3305, 3224, 3210, 3165, 1453; δ_H (600 MHz, CD_3OD, Me_4Si) [*trans*- isomer data listed only] 7.48 (d, J = 9.8, 1H), 7.53-7.60 (m, 3H), 7.96 (d, J = 6.8, 2H), 8.07 (d, J = 8.3, 2H), 8.18 (d, J 8.3, 2H), 8.33 (d, J = 9.8, 1H); δ_C (150 MHz, CD_3OD) 121.6 (CH), 124.0 (CH), 124.5 (CH), 128.1 (C), 128.5 (CH), 130.0 (C), 130.4 (CH), 132.8 (CH), 154.0 (C), 154.8 (C), 173.1 (C) {1 CH signal unobserved}; *m/z* (ES^+) 276 (100%, $[M+H]^+$); HRMS (ES^+) $C_{16}H_{14}N_5^+$ ($[M+H]^+$) requires 276.1242, measured 276.1245.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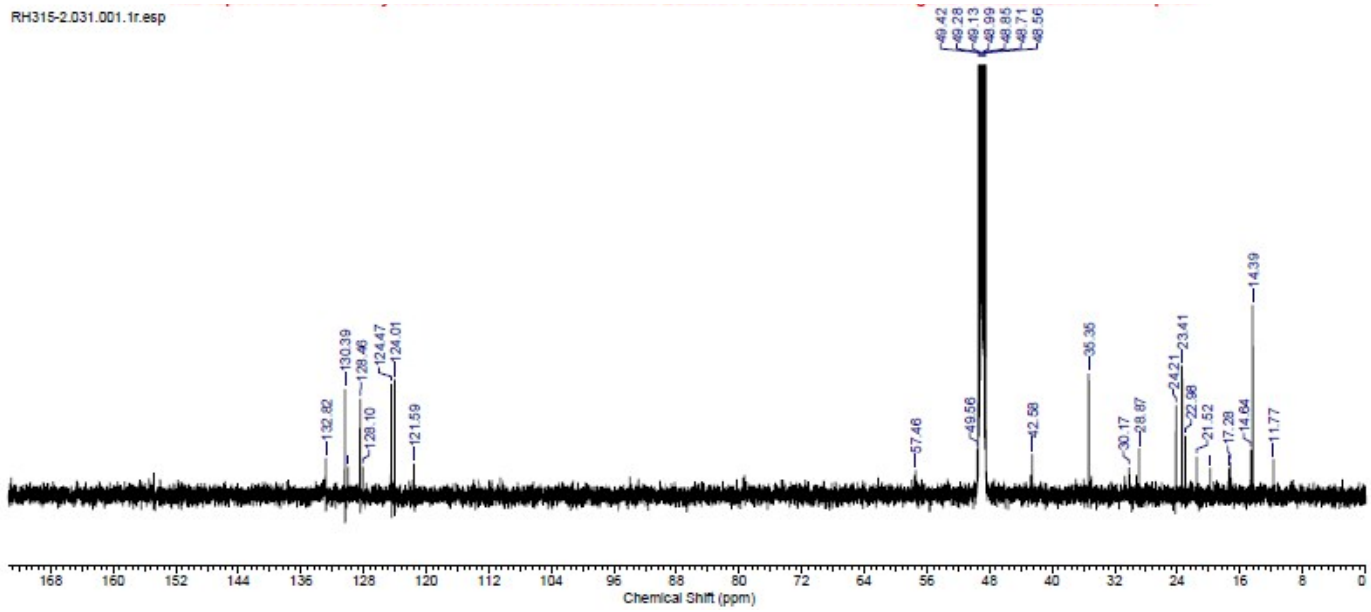
Minor isomer: *cis*-**3**
Chemical Shift (ppm)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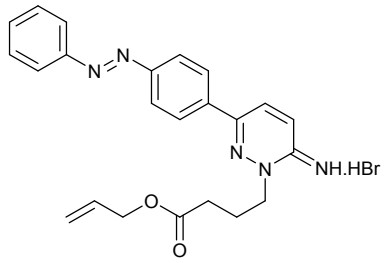
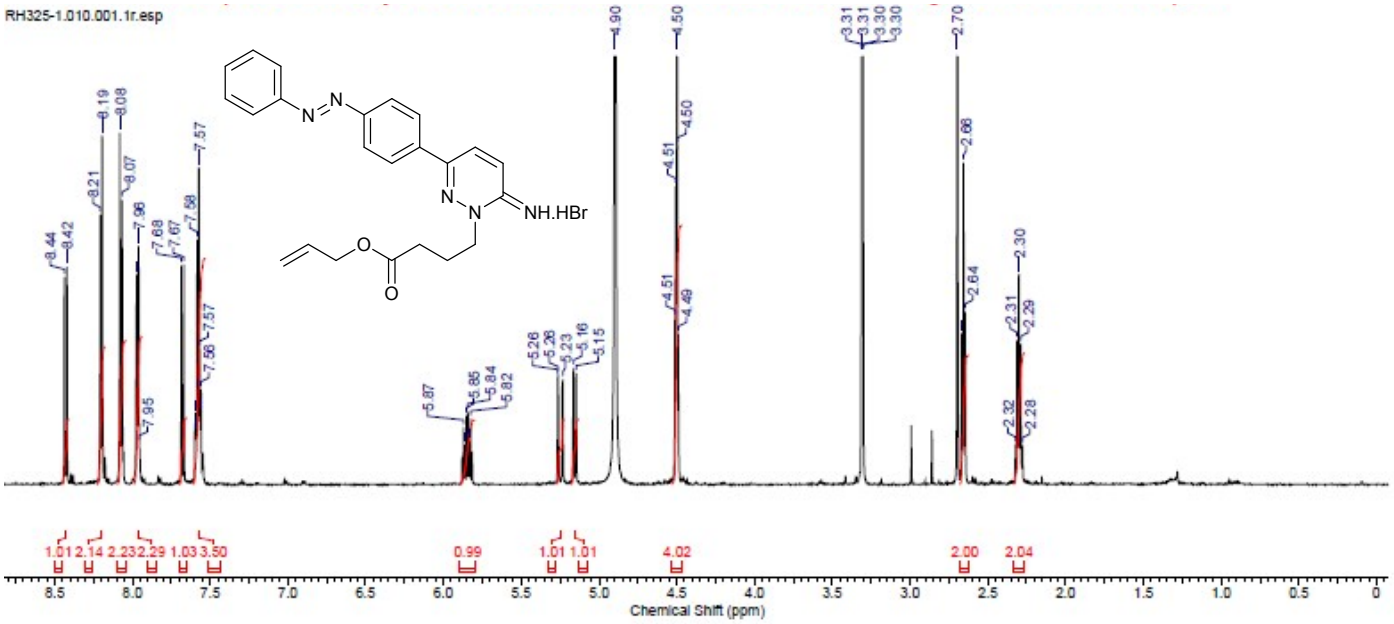
Chemical Shift (ppm)

Allyl 4-(6-imino-3-(4-(phenyldiazenyl)phenyl)pyridazin-1(6H)-yl)butanoate, 4.



A mixture of **3** (14 mg, 0.051 mmol) and allyl 4-bromobutyrate (10.5 μL, 0.051 mmol) in DMF (0.1 mL) was heated at 80 °C for 16 h. After this time EtOAc (5 mL) was added and the reaction cooled to 0 °C. The resulting precipitate was filtered and washed with cold EtOAc (2 mL) to give **4** (14.9 mg, 73%) as a yellow solid.

mp 169-170 °C (from CH_3OH); ν_{max} (solid) 3319, 3040, 1735; δ_H (600 MHz, CD_3OD, Me_4Si) 2.30 (quin, J = 6.8, 2H), 2.66 (t, J = 6.8, 2H), 4.48-4.52 (m, 4H), 5.16 (dd, J = 10.4, 1.5, 1H), 5.25 (dd, J = 17.3, 1.5, 1H), 5.81-5.88 (m, 1H), 7.54-7.60 (m, 3H), 7.68 (d, J = 9.4, 1H), 7.95-7.98 (m, 2H), 8.08 (d, J = 8.3, 2H), 8.20 (d, J = 8.3, 2H), 8.43 (d, J = 9.4, 1H); δ_C (125 MHz, CD_3OD) 22.5 (CH_2), 31.3 (CH_2), 57.1 (CH_2), 66.5 (CH_2),118.6 (CH_2), 124.1 (CH), 124.5 (CH), 127.0 (CH), 128.8 (CH),130.4 (CH), 132.8 (CH), 133.0 (CH), 133.4 (CH), 136.3 (C), 151.3 (C), 154.0 (C), 154.4 (C), 155.2 (C), 174.1 (C); *m*/*z* (ES^+) 402 (100%, $[M+H]^+$); HRMS (ES^+) $C_{23}H_{24}N_5O_2$ ($[M+H]^+$) requires 402.1930, measured 402.1892.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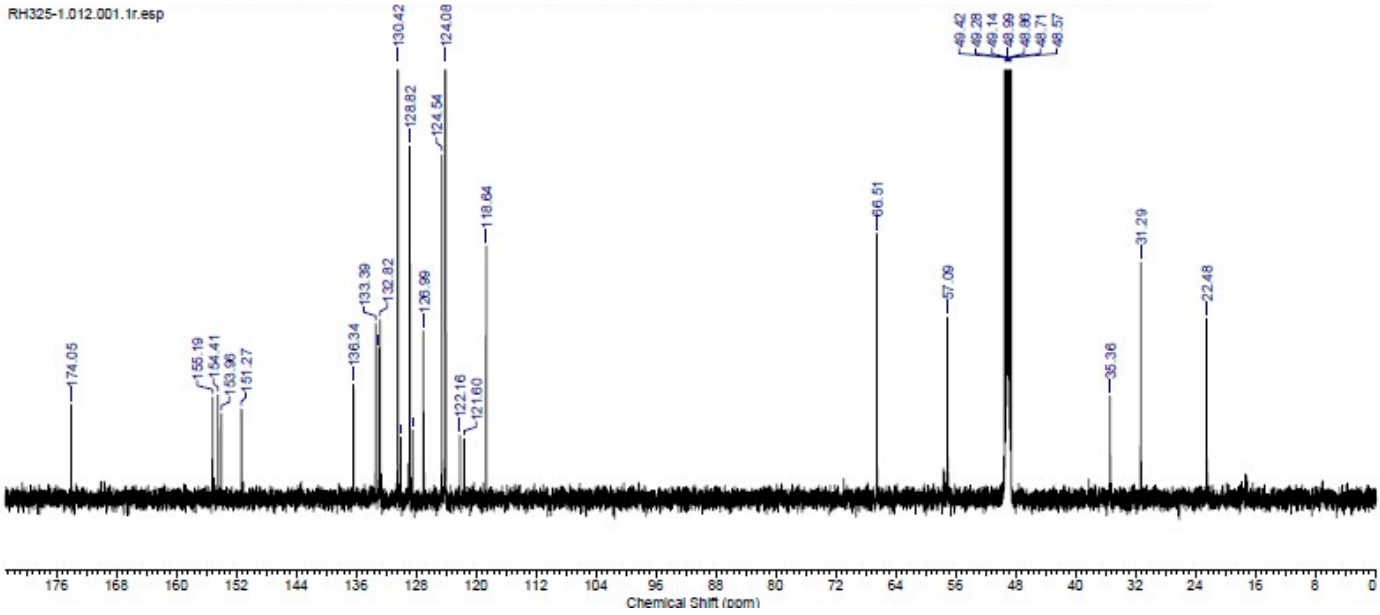
NH.HBr
Chemical Shift (ppm)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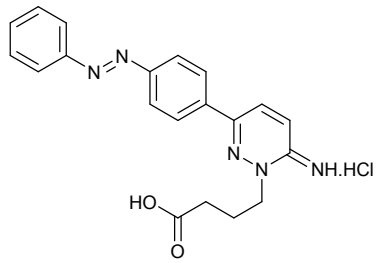
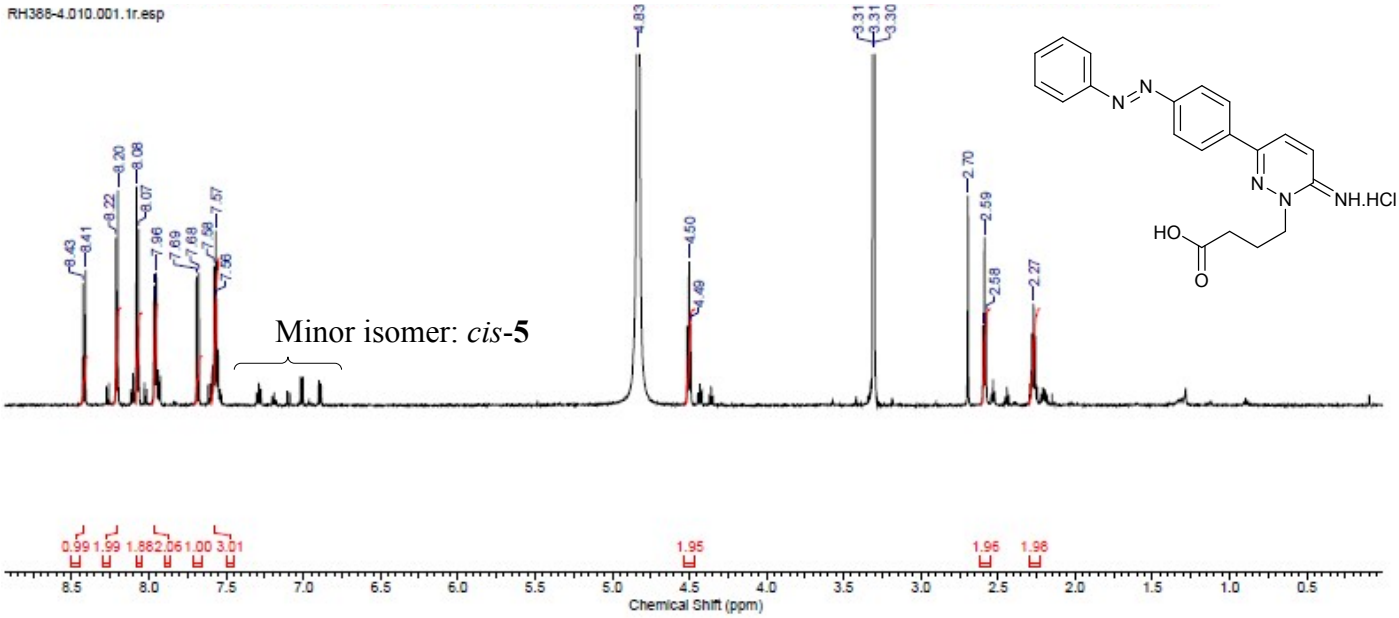
Chemical Shift (ppm)

4-(6-Imino-3-(4-(phenyldiazenyl)phenyl)pyridazin-1(6H)-yl)butanoic acid {azo-gabazine}, 5.



A solution of NaOH (20 mg) and ester **4** (14.9 mg, 0.037 mmol) in THF (1 mL) and H_2O (1 mL) was stirred at 50 °C for 3 h. The mixture was then cooled to 10 °C and washed with EtOAc (3 mL). The aqueous layer was acidified to pH 1 with 1 M aqueous HCl and stirred at rt for 1 h. The solvent was then concentrated *in vacuo*, and the residue triturated with H_2O (2 mL) to yield **5** (12.3 mg, 80%) as a yellow solid.

mp 242-246 °C (from EtOH); ν_{max} (solid) 3039, 1718; δ_H (600 MHz, CD_3OD, Me_4Si) [*trans*- isomer data listed only] 2.26 (quin, *J* = 6.9, 2H), 2.59 (t, *J* = 6.9, 2H), 4.50 (t, *J* = 7.1, 2H), 7.50-7.60 (m, 3H), 7.70 (d, *J* = 9.4, 1H), 7.96 (d, *J* = 7.5, 2H), 8.07 (d, *J* = 7.9, 2H), 8.21 (d, *J* = 8.3, 2H), 8.42 (d, *J* = 9.4, 1H); δ_C (150 MHz, CD_3OD, Me_4Si) 22.6 (CH_2), 31.0 (CH_2), 57.1 (CH_2), 124.1 (CH), 124.5 (CH),126.9 (CH), 128.9 (CH),130.4 (CH), 132.8 (CH), 133.0 (CH), 136.4 (C), 154.0 (C), 154.4 (C), 155.2 (C), 176.4 (C), 190.5 (C); *m*/*z* (ES^+) 362 (100%, $[M+H]^+$); HRMS (ES^+) $C_{20}H_{19}N_5O_2^+$ ($[M]^+$) requires 362.1617; measured 362.1618.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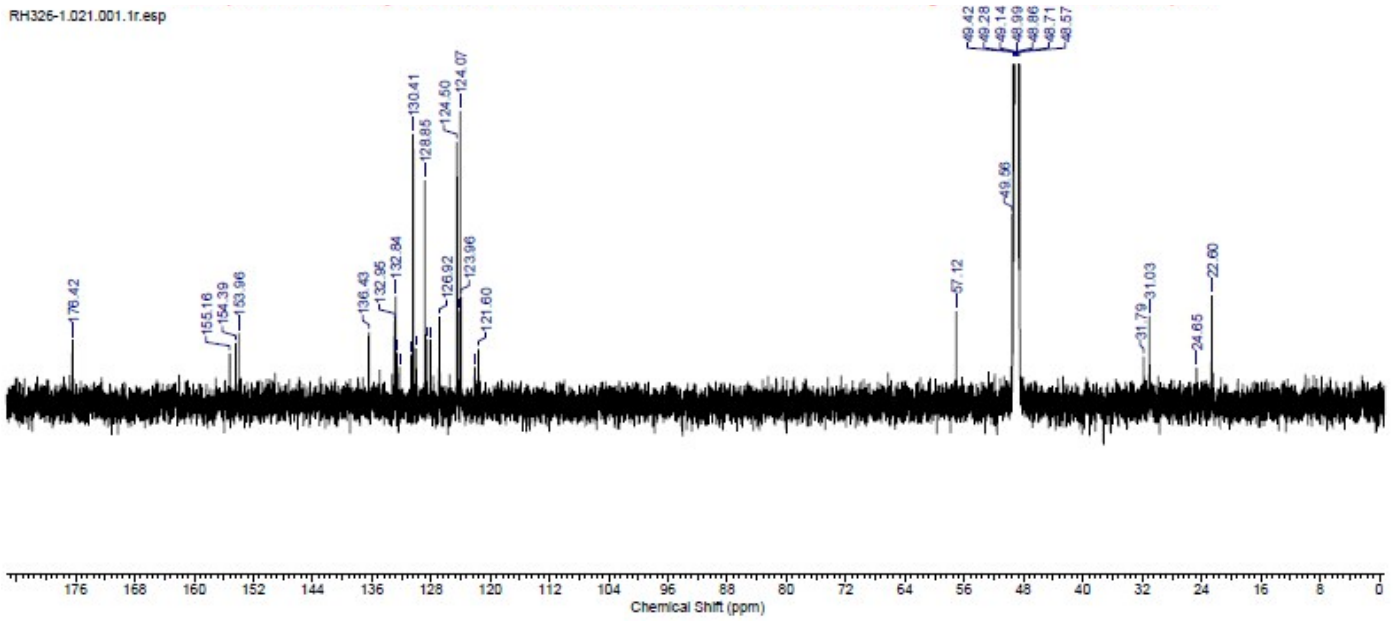
Minor isomer: *cis*-**5**
NH.HCl
HO
O
Chemical Shift (ppm)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Chemical Shift (ppm)

Electrophysiology methods

HEK cells were maintained at 37°C, 95% CO_2/5% O_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 100 u/ml penicillin-G and 100 μg/ml streptomycin. Cells were transfected with cDNAs encoding enhanced green fluorescent protein (EGFP) and murine α1, β2, γ2S $GABA_A$ receptor subunits (ratio: 1:1:1:1) using a standard calcium-phosphate transfection protocol[2]. Cells were left to express EGFP and $GABA_A$ receptors for 16 - 24 hrs before electrophysiology.

Whole-cell patch clamp recording from HEK cells was used to monitor GABA currents as described previously[3] using an Axopatch 200B (Molecular Devices) amplifier. Patch pipettes (resistance 3 – 5 MΩ) were filled with a solution containing (mM): 120 KCl, 1 $MgCl_2$, 11 EGTA, 30 KOH, 10 HEPES, 1 $CaCl_2$, and 2 adenosine triphosphate; pH 7.11. The cells were continuously perfused with Krebs recording solution containing (mM): 140 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 2.52 $CaCl_2$, 11 Glucose and 5 HEPES; pH 7.4. Drugs made up in Krebs solution were applied via a Y-tube application system.

For competitive inhibition experiments, azogabazine (a range of concentrations) was pre-applied to cells for 5 - 10 s before co-application with 10 μM GABA (~EC_{50} on α1β2γ2 $GABA_A$ receptors). The potency of azogabazine inhibition was evaluated by plotting the inhibition-concentration relationship for each experiment and fitting the data with the following equation: $I/I_{max} = 1 - [1/1 + (IC_{50}/B)^n)]$, where the IC_{50} is the antagonist concentration (B) causing half-maximal inhibition of the GABA induced response[1]. The IC_{50} values obtained from 6 individual experiments were converted to pIC_{50} (-Log IC_{50}) from which a mean pIC_{50} ± sem was calculated. The mean value was then converted back into the reported mean IC_{50} value.

Photo-isomerisation was performed using a TTL controlled Cairn Optoflash 365 nm UV LED and Cairn Optoled 470 nm blue LED directing light to the recorded cell via Nikon microscope optics and a 40x water-dipping objective and through ~3.5 mm recording solution. Measurement of optical power was undertaken using an energy meter (ThorLabs PM200 + Si Photodiode - FDS10X10): 0.28 mW.

The blue and UV light exposures were applied in the following protocol: 3s blue, 3s UV, 15s blue, 3s UV, 15s blue, 3s UV. A control light protocol run in Krebs recording solution in the absence of GABA and azogabazine did not induce any discernible current whereas co-application of GABA and azogabazine led to GABA-

induced currents with UV, presumably from azogabazine unbinding and GABA binding causing receptor activation.

Irradiation of Azogabazine - NMR experiment set-up

To conduct ^{1}H NMR analysis, a solution of azogabazine (10 mM in DMSO-d_6) was diluted ten-fold with deuterium oxide, to give a 1 mM solution. In order to try to mimic our electrophysiology experimental conditions, and to achieve effective sample irradiation, an LED (365 nm or 470 nm OptoLED from Cairn Research) was clamped ~1.5 cm above a 0.7 mL droplet of the 1 mM solution as it rested on a square of Parafilm "M" laboratory film. Energy outputs were 6.3 mW and 9.7 mW from the 365 nm (UV) or 470 nm (blue) LEDs, respectively (measured using a ThorLabs PM200 optical energy meter with an FDS10X10 Si Photodiode). After irradiation the sample was transferred via syringe to an NMR tube, which was then placed onto the carousel and loaded into the NMR machine by automation. In general the NMR tube was exposed to ambient light for ~ 20 seconds before reaching darkness inside the instrument, and a complete spectrum was obtained in ~ 4 minutes. Integration of peaks assigned for each isomer gives the isomeric ratio (Figure (i)).

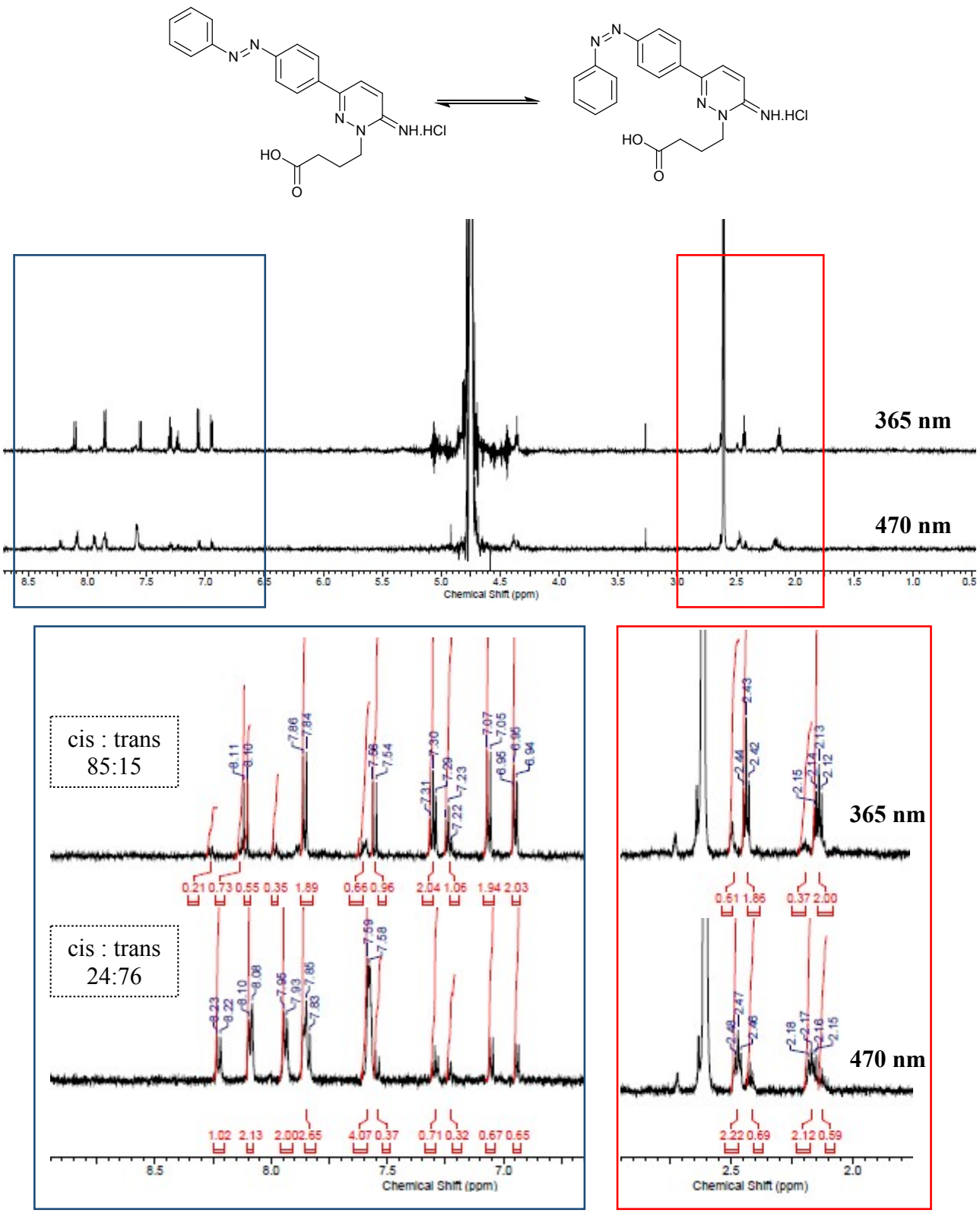

Figure (i): NMR spectrum obtained after irradiation with 365 nm (top) and 470 nm (bottom) LEDs for 60 seconds, showing conversion to *cis*- and *trans*- enriched photostationary states respectively.

Irradiation of Azogabazine – for UV spectra

150uL of stock azogabazine solution (27 mM in MeCN) was placed in a quartz cuvette and a UV spectrum was run on a Cary UV-Vis spectrophotometer. The cuvette was then removed and the sample was irradiated for 30 s with a 365nm hand-held torch (5W LED torch from Advanced NDT Ltd) which rested on top of the cuvette. The cuvette was returned to the spectrophotometer and a further spectrum was recorded.

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[1] J. H. Harvey, B. K. Butler, D. Trauner, *Tet. Lett.*, 2007, **48**, 1661-1664.
[2] M. Mortensen, B. Patel, T. G. Smart, *Front Cell Neurosci,* 2011, **6**, 1-10.
[3] M. Mortensen, U. Kristiansen, B. Ebert, B. Frølund, P. Krogsgaard-Larsen, T. G. Smart, *J Physiol*. 2004 **557**, 393–417.