

A Photoswitchable Inhibitor of the Calcium Channel TRPV6

Micael R. Cunha,[†] Rajesh Bhardwaj,[±] Sonja Lindinger,[‡] Carmen Butorac,[‡] Christoph Romanin,^{*‡}
Matthias A. Hediger,^{*±} Jean-Louis Reymond^{*†}

[†]Department of Chemistry and Biochemistry, NCCR TransCure, University of Bern, Freiestrasse 3, 3012 Bern.

[±]Institute of Biochemistry and Molecular Medicine, NCCR TransCure, University of Bern, Bülhlstrasse 28, 3012 Bern, Switzerland & Department of Nephrology and Hypertension, University Hospital Bern, Inselspital, 3010 Bern, Switzerland

[‡]Institute of Biophysics, Johannes Kepler University Linz, Gruberstrasse 40, 4020 Linz.

†SUPPORTING INFORMATION

Table of contents

1. General methods.....	2
2. Synthetic chemistry, procedure and physical properties.....	3
3. Copy of ¹ H and ¹³ C NMR spectra	11
4. X-Ray crystal structure report.....	31
5. Photochemical properties of 9a-e.....	32
6. Analytical RP-UPLC purity for compounds 9a-e	34
7. Biological methodology	34
8. References	37

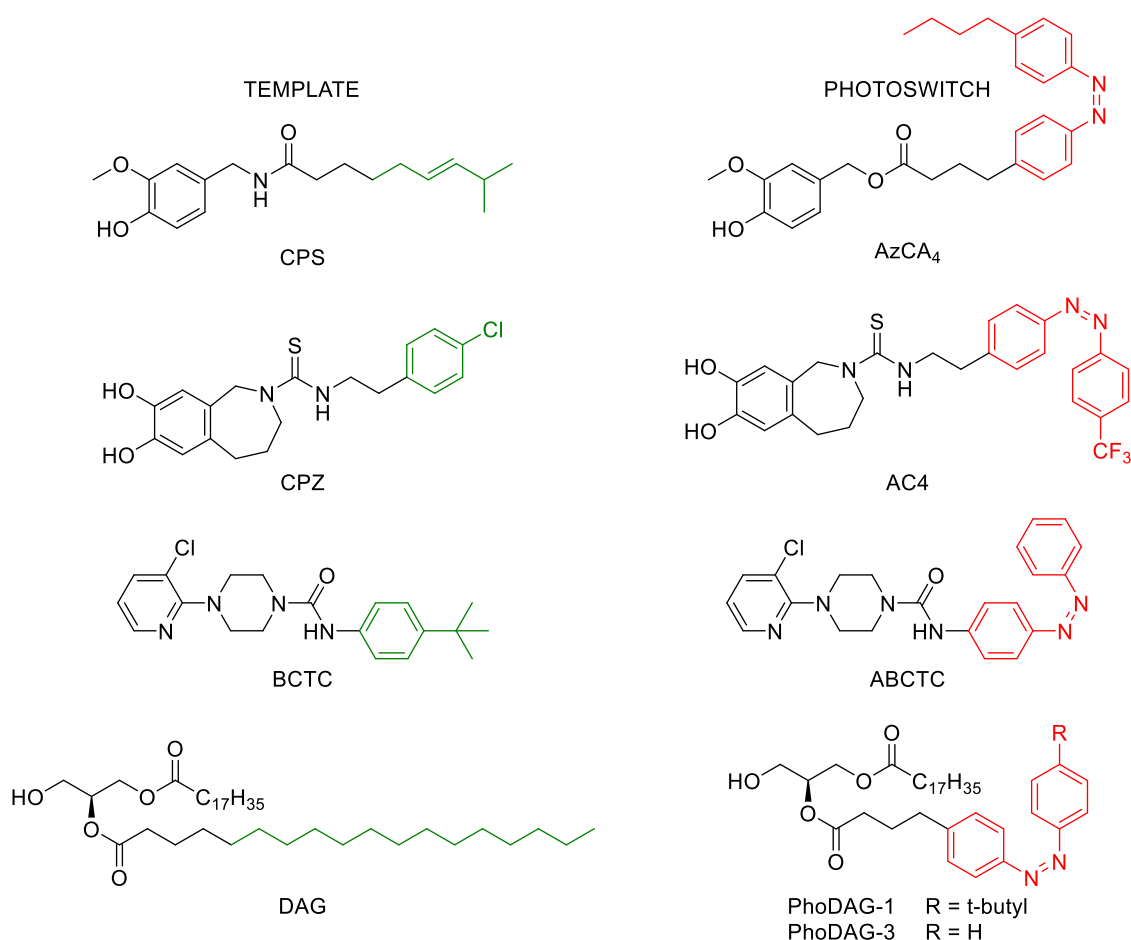


Figure S1. On the left side, structures of the bioactive compounds used as templates to generate photoswitches. On the right side, the phenyldiazo group is depicted in the active form (Z). CPS = capsaicin, TRPV activator; CPZ = capsazepine, TRPV1 inhibitor; BCTC = N-(4-Tert-butylphenyl)-4-(3-chloropyridin-2-yl)piperazine-1-carboxamide; TRPV1 inhibitor; DAG = diacylglycerol; TRPC2/3/6 activator.

1. General methods.

All commercial reagents were used without further purification. Dry solvents were obtained directly from a drying solvent system. Chromatographic purifications were performed using silica gel (Sigma-Aldrich, 230-400 mesh). Automated chromatographic purification was performed with Puriflash 430 system (Interchim) using Teledyne Isco normal phase RediSepRf cartridge and detection by UV absorption (214 nm). Low resolution mass spectra were obtained by electron spray ionization (ESI), positive mode (Thermo Scientific LCQ Fleet). High resolution mass spectra were obtained by ESI, positive mode (Thermo Scientific LTQ OrbitrapXL). ¹H and ¹³C-NMR spectra were

recorded at 300MHz and 75MHz, respectively (Bruker AV300). ^1H and ^{13}C chemical shifts are quoted relative to solvent signals. MestreNova was used for further analysis of the spectra. The purity of all tested compounds was >95% (**Table S13**), as confirmed by analytical RP-UHPLC with detection at 214nm, on a Dionex Ultimate 3000 RSLC System (DAD-3000 RS Photodiode Array Detector) and Dionex Acclaim RSLC 120 column (C18, 3.0 x 50 mm, particle size 2.2 μm , 120Å pore size) at a flow rate of 1.2 mL/min. Data recording and processing was done with Dionex Chromelon Management System (v. 6.8), and Xcalibur (v. 2.2, Thermo Scientific). Eluents for analytical RP-UHPLC were as follow: A: miliQ-deionized water with 0.05% TFA and D: HPLC-grade acetonitrile/miliQ-deionized water (9/1) with 0.05% TFA. Conditions for analytical RP-UHPLC were as follow: in 4.5 min from 100% A to 100% D, then staying on 100% D, or in 7.5 min from 100% A to 100% D, then staying on 100% D. Chemical names were generated using ChemDraw Professional 17.0 (PerkinElmer Informatics).

2. Synthetic chemistry, procedure and physical properties.

(E)-1-(6-(phenyldiazenyl)pyridin-3-yl)piperazine, double hydrochloride salt (5a).

To a warm solution (60 °C) of NaOH (60.0 mmol, 2.40 g) in a H_2O (4.7 mL), was added a solution of **3a** (5.00 mmol, 1.39 g) in benzene (0.564 mL). After become soluble, nitrosobenzene (5.00 mmol, 535.5 mg) was added in small portions. After addition, the reaction was refluxed (100 °C) for 10 min. Once reached r.t., the mixture was diluted with benzene (10.0 mL) and extracted with H_2O (2x10 mL). The collected aqueous phase was washed with 10.0 mL of benzene. The organic phases were collected, dried over MgSO_4 , and concentrated over vacuum. Column chromatography (Hexanes 5:5 AcOEt) afforded the **4a** as an orange solid (835.5 mg, 2.27 mmol, 45%). The previously obtained compound was suspended an aqueous solution of HCl (1 M, 100.0 mL) was refluxed (100 °C) o.n. After completion, the solvent was partially removed by vacuum (until 10.0 mL) to give **5a** as an orange powder (647.6 mg, 2.13 mmol, 84%). ^1H -NMR (300 MHz, D_2O , δ = ppm): δ 7.80 (s, 1H), 7.53-7.36 (m, 7H), 3.63 (s, 4H), 3.46 (s, 4H). ^{13}C -NMR (75 MHz, D_2O , δ = ppm): δ 150.5, 147.4, 144.9, 133.7, 129.6, 128.4, 126.5, 123.5, 119.5, 117.5, 43.1, 42.5, 40.3. HRMS m/z calculated for $\text{C}_{15}\text{H}_{18}\text{N}_5$: 268.1557 ($[\text{M}+\text{H}]^+$); found: 268.1563.

(E)-1-(4-(phenyldiazenyl)phenyl)piperazine, hydrochloride salt (5b). To a solution of **3b** (5.00 mmol, 1.54 g) in AcOEt (50.0 mL) under Argon, was added Pd/C 10 %. The suspension was stirred vigorously under an atmosphere of H_2 (balloon) for 48 h

at r.t. The mixture was filtered through Celite, which was washed several times with AcOEt. The filtrate was concentrated in vacuum to give the crude amine derivative as a pale pink solid. The crude amine was solubilized in AcOH (10.0 mL) and was added to a solution of nitrosobenzene (5.00 mmol, 536 mg; AcOH, 10 mL). The reaction mixture was stirred at r.t. for 24 h until completion (monitored by TLC). The solvent was removed by vacuum, and the crude was purified by column chromatography (hexanes 7:3 AcOEt) to afford **4b** as an orange solid (1.12 g, 3.07 mmol, 61% over 2 steps). A suspension of the previous obtained compound (3.07 mmol, 1.12 g) in an aqueous solution of HCl (1 M, 250.0 mL) was refluxed (100 °C) o.n. After completion, the solvent was partially removed by vacuum (until 10.0 mL). Compound **5b** was obtained as a dark-purple solid (hydrochloride salt) after lyophilization (929.0 mg, 3.07 mmol, 61% over 3 steps). ¹H-NMR (300 MHz, CD₃OD, δ = ppm): δ 7.91 (dt, J = 3.1, 2.1 Hz, 2H), 7.86 (dt, J = 2.1, 1.6 Hz, 2H), 7.50 (m, 3H), 7.18 (dt, J = 3.1, 2.1 Hz, 2H), 3.62 (t, J = 5.5, 5.0 Hz, 4H), 3.41 (t, J = 5.5, 5.0 Hz, 4H). ¹³C-NMR (75 MHz, CD₃OD, δ = ppm): δ 142.1, 131.5, 130.2, 125.6, 123.4, 116.9, 46.7, 44.6. HRMS m/z calculated for C₁₆H₁₉N₄: 267.1604 ([M+H]⁺); found: 267.1603.

(E)-(4-piperazin-1-ylphenyl)-(3-pyridyl)diazene, double hydrochloride salt (5c). A solution of **3c** (8.09 mmol, 761.0 mg) in a mixture (1:1) of HCl/H₂O (55.0 mL), was stirred in an ice bath. Then, a solution of NaNO₂ (7.35 mmol, 507.0 mg, in H₂O, 5.0 mL) was added drop-by-drop to the previous suspension, and the mixture was stirred at 0 °C, to generate *in situ* **4c**. After 30 min, phenylpiperazine (**7**) was added, drop-by-drop, and the mixture was allowed to stir o.n. at r.t. The reaction was quenched by addition of a saturated solution of NaHCO₃ until pH aprox. 8.0. The solids were filtered off, washed with H₂O (10.0 mL), and solubilized in an aqueous solution of HCl (5.0 %). The solvent was removed by vacuum. The crude was solubilized in dry MeOH and the compound was precipitated by addition of dry diethyl ether. The solids were collected, washed with hexanes, dried in vacuum. The title compound was solubilized in H₂O and lyophilized to give **5c** as a purple powder (1.63 g, 4.79 mmol, 65% over 2 steps). ¹H-NMR (300 MHz, CD₃OD, δ = ppm): δ 9.31 (d, J = 2.0 Hz, 1H), 8.98-8.94 (ddd, J = 8.4, 2.0, 1.4 Hz, 1H), 8.89 (d, J = 5.5 Hz, 1H), 8.22 (dd, J = 8.4, 5.7 Hz, 1H), 8.03 (d, J = 9.2 Hz, 2H), 7.21 (d, J = 9.2 Hz, 2H), 3.76 (t, J = 5.4 Hz, 4H), 3.41 (t, J = 5.1, 5.4 Hz, 4H). ¹³C-NMR (75 MHz, CD₃OD, δ = ppm): δ 155.3, 151.9, 146.6, 142.7, 138.9, 138.3, 129.3, 127.4, 116.1, 45.7, 44.4. HRMS m/z calculated for C₁₅H₁₈N₅: 268.1557 ([M+H]⁺); found: 268.1562.

(6-aminopyridin-3-yl)methanol (**4d**). A suspension of LiAlH_4 in THF (12.0 mmol, 12.0 mL) was stirred at 0 °C under argon atmosphere. To this solution, **3d** (10.0 mmol, 1.52 g) in anhydrous THF (10.0 mL) was added dropwise. The reaction mixture was allowed to warm to r.t. and stirred for 24h. The system was cooled to 0 °C and water (3.0 mL) was added slowly, followed by 10% NaOH (3.0 mL), and additional water (1.5 mL). The salts were removed by filtration through Celite, which was washed several times with THF. The resulting solution was dried over MgSO_4 and evaporated by vacuum. The crude was column chromatographed (CH_2Cl_2 75:25 MeOH +0.1% Et_3N) to give **4d** as a white powder (945.0 mg, 7.61 mmol, 76%). ^1H -NMR (300 MHz, CD_3OD , δ = ppm): δ 7.94 (d, J = 8.8 Hz, 1H), 7.82 (s, 1H), 7.07 (d, J = 9.0 Hz, 1H) 5.16 (s, 3H), 4.53 (s, 2H). ^{13}C -NMR (75 MHz, CD_3OD , δ = ppm): δ 155.2, 145.1, 133.8, 128.1, 114.7, 60.9. HRMS m/z calculated for $\text{C}_6\text{H}_9\text{N}_2\text{O}$: 125.0709 ($[\text{M}+\text{H}]^+$); found: 125.0700.

5-(((*tert*-butyldimethylsilyl)oxy)methyl)pyridin-2-amine (**p-4d**). **4d** (7.60 mmol, 945.0 mg) was dissolved in a mixture of CH_2Cl_2 :DMF (50.0 mL; 4:1). The mixture was stirred at 0 °C and DIPEA (11.4 mmol, 2.00 mL), TBDMSCl (10.6 mmol, 1.60 g), and DMAP (0.15 mmol, 18.6 mg) were added. The reaction was allowed to warm to r.t., and stirred for 8h. The solvents were removed by vacuum. The crude was column chromatographed (Hexanes 5:5 AcOEt) to give **p-4d** as a white powder (968.0 mg, 4.06 mmol, 53%). ^1H -NMR (300 MHz, CDCl_3 , δ = ppm): δ 7.98 (d, J = 1.7 Hz, 1H), 7.40 (dd, J = 8.4, 2.2 Hz, 1H), 6.46 (d, J = 8.4 Hz, 1H) 4.57 (s, 2H), 4.46 (s, 2H), 0.89 (s, 9H), 0.06 (s, 6H). ^{13}C -NMR (75 MHz, CDCl_3 , δ = ppm): δ 157.9, 146.5, 137.1, 126.7, 108.5, 62.9, 26.0, 18.4, -5.1. HRMS m/z calculated for $\text{C}_{12}\text{H}_{23}\text{N}_2\text{OSi}$: 239.1574 ($[\text{M}+\text{H}]^+$); found: 239.1568.

(*E*)-5-(((*tert*-butyldimethylsilyl)oxy)methyl)-2-(phenyldiazenyl)pyridine (**p-5d**). To a solution of **p-4d** (2.22 mmol, 530.0 mg) in toluene (0.88 mL), a solution of NaOH (26.7 mmol, 1.07 g) in H_2O (2.20 mL) followed by nitrosobenzene (2.67 mmol, 286.0 mg) was added. The reactional mixture was vigorously stirred at 50 °C for 17 h. After cooling to r.t., H_2O (1.50 mL) was added and the mixture was extracted with CH_2Cl_2 (3 X 20.0 mL). The combined organic phases were dried in Na_2SO_4 , and the solvents removed by vacuum. The crude was column chromatographed (Hexanes 9:1 AcOEt to Hexanes 8:2 AcOEt) to afford **p-5d** as a red powder (402.0 mg, 1.23 mmol, 55%). ^1H -NMR (300 MHz, CDCl_3 , δ = ppm): δ 8.68 (d, J = 1.3 Hz, 1H), 8.05 (m, 2H), 7.88 (dd, J = 8.2, 2.0 Hz, 1H), 7.83 (d, J = 8.1 Hz, 1H), 7.57-7.52 (m, 3H), 4.86 (s, 2H), 0.95 (s, 9H), 0.13 (s, 6H). ^{13}C -NMR (75 MHz, CDCl_3 , δ = ppm): δ 162.2, 152.6, 147.6, 138.9, 136.3,

132.1, 129.3, 115.6, 62.6, 26.0, 18.5, -5.1. HRMS m/z calculated for $C_{18}H_{26}N_3OSi$: 328.1840 ($[M+H]^+$); found: 328.1841.

(*E*)-(6-(phenyldiazenyl)pyridin-3-yl)methanol (**5d**). Finally, to a solution of **p-4d** (1.23 mmol, 402.0 mg) in THF (3.80 mL), was added a solution of TBAF in THF (2.45 mmol, 3.7 mL). The reactional mixture was stirred at r.t. for 2 h. After completion (monitored by TLC), the reaction was quenched by the addition of saturated solution of $NaHCO_3$ (3.0 mL). The mixture was then extracted with AcOEt (2 x 10.0 mL). The collected organic phase was washed with H_2O (2 x 10.0 mL), followed by Brine (2 x 10.0 mL). After drying in Na_2SO_4 , the solvents were removed by vacuum to afford the **5d** as an orange powder (236.0 mg, 1.11 mmol, 90%) used in the next step without further purification. 1H -NMR (300 MHz, $CDCl_3$, δ = ppm): δ 8.61 (d, J = 1.4 Hz, 1H), 7.98 (dd, J = 6.6, 2.9 Hz, 2H), 7.88 (dd, J = 8.2, 1.9 Hz, 1H), 7.75 (d, J = 8.2 Hz, 1H), 7.50 (t, J = 3.6, 2.8 Hz, 3H), 4.80 (s, 2H), 3.74 (s, 1H). ^{13}C -NMR (75 MHz, $CDCl_3$, δ = ppm): δ 162.1, 152.4, 147.8, 138.9, 137.2, 129.3, 123.6, 115.1, 62.0. HRMS m/z calculated for $C_{12}H_{12}N_3O$: 214.0975 ($[M+H]^+$); found: 214.0978.

(*E*)-1-phenyl-2-(*p*-tolyl)diazene (**4e**). To a suspension of **3e** (4.50 mmol, 482.0 mg) in AcOH (5.0 mL) was added a suspension of nitrosobenzene (4.50 mmol, 282.0 mg) in AcOH (5.0 mL). The reaction suspension was stirred vigorously for 24 h at r.t. The solvent was removed by vacuum. The crude compound was column chromatographed (Hexanes 7:3 AcOEt) to afford **4e** as a yellow powder (822.0 mg, 4.19 mmol, 93%). 1H -NMR (300 MHz, $CDCl_3$, δ = ppm): δ 7.91 (dd, J = 8.2, 1.3 Hz, 2H), 7.85 (d, J = 8.3 Hz, 1H), 7.55-7.42 (m, 3H), 7.32 (d, J = 8.1 Hz, 1H), 2.45 (s, 3H). ^{13}C -NMR (75 MHz, CD_3OD , δ = ppm): δ 150.9, 141.7, 130.8, 129.9, 129.2, 123.0, 122.9, 21.6. HRMS m/z calculated for $C_{13}H_{13}N_2$: 197.1073 ($[M+H]^+$); found: 197.1080.

(*E*)-1-(4-(bromomethyl)phenyl)-2-phenyldiazene (**5e**).

To a solution of **4e** (4.19 mmol, 822.0 mg) and NBS (4.20 mmol, 748.0 mg) in CCl_4 (15.0 mL) was added BPO (0.006 mmol, 6.1 mg). The reaction mixture was stirred vigorously under reflux and in an atmosphere of argon for 24 h. The solids were filtered off and washed with CCl_4 . The solvent was removed by vacuum (2 additional traps) to give a mixture of **5e** and (*E*)-[4-(bromomethyl)phenyl]-phenyl-diazene. The crude mixture was column chromatographed (petrolether) to afford the desired azobenzene **5e** as an orange powder (1.14 g, 4.15 mmol, 99%) used in the next step without further purification. 1H -NMR (300 MHz, $CDCl_3$, δ = ppm): δ 7.95-7.92 (m, 4H), 7.57-7.46 (m, 5H), 4.56 (s, 2H). ^{13}C -NMR (75 MHz, CD_3OD , δ = ppm): δ 152.7, 152.5, 140.6, 131.4,

130.0, 129.2, 123.4, 123.1, 32.9. HRMS m/z calculated for $C_{13}H_{12}BrN_2$: 275.0178 ($[M+H]^+$); found: 275.0179.

1-(4-phenylcyclohexyl)piperazine, double hydrochloride salt (8). To a solution of 4-phenylcyclohexanone (**6**, 10.0 mmol, 1.74 g) and tert-butyl piperazine-1-carboxylate (11.0 mmol, 2.05 g) in dry DCE (20.0 mL), was added AcOH (1.75 mmol, 0.10 mL). The reaction mixture was stirred at r.t. for 6h. After complete consumption of the amine (TLC), $NaBH(OAc)_3$ (13.0 mmol, 2.75 g) was added in small portions. The mixture was stirred for 48 h at r.t. The reaction was quenched by addition of saturated solution of $NaHCO_3$ (20.0 mL). The mixture was extracted with CH_2Cl_2 (3 x 20.0 mL). The organic phase was collected and extracted with an aqueous solution of HCl (1M; 3 x 20.0 mL) and was washed with H_2O (1 x 20.0 mL). The resulting organic phase was dried in Na_2SO_4 , and the solvent was removed by vacuum to afford the desired boc-protected amine as a white powder and was used in the next step without further purification. The amine was refluxed (90 °C) in an aqueous HCl solution (2.0 M; 35.0 mL) for 90 min. The solvent was partially removed by vacuum. The resulting solution was diluted with H_2O (10.0 mL) and lyophilized to afford **8** as a mixture of diastereomers of 1-(4-phenylcyclohexyl)piperazine (white powder, 2.79 g, 8.78 mmol, 88%) used in the next step without further purification. 1H -NMR (300 MHz, D_2O , δ = ppm): δ 7.37-7.24 (m, 5H), 3.74-3.43 (m, 9H), 2.84 (s, 1H), 2.25-1.30 (m, 8H). ^{13}C -NMR (75 MHz, $CDCl_3$, δ = ppm): δ 145.8, 144.3, 128.7, 127.4, 126.9, 126.2, 65.8, 65.4, 46.0, 45.5, 41.0, 40.5, 37.9, 31.6, 27.3, 26.4, 23.5. HRMS m/z calculated for $C_{16}H_{25}N_2$: 245.2012 ($[M+H]^+$); found: 245.2011.

1-((1S,4S)-4-phenylcyclohexyl)-4-(6-((E)-phenyldiazenyl)pyridin-3-yl)piperazine, double hydrochloride salt (9a). To a solution of **5a** (1.10 mmol, 374.2 mg) and **6** (1.00 mmol, 174.2 mg) in dry DCE (5.00 mL) was added Et_3N (2.10 mmol, 0.292 mL). The solution was stirred vigorously at r.t. for 3 h. $NaBH(OAc)_3$ (1.30 mmol, 274.3 mg) was added in small portions and the mixture was stirred for 2 days at r.t. The solvent was removed in vacuum. The crude was subjected to column chromatography (Hexanes 7:3 AcOEt + 0.5% Et_3N to Hexanes 5:5 AcOEt + 0.5% Et_3N). The title compound was solubilized in a mixture a methanolic solution of HCl (1.25 M) and precipitated by the addition of dry diethylether to afford **9a** as an orange powder (198.8 mg, 0.40 mmol, 40%). 1H -NMR (300 MHz, C_6D_6 , δ = ppm): δ 8.41 (d, J = 3.0 Hz, 1H), 8.23-8.19 (m, 2H), 7.94 (d, J = 9.0 Hz, 1H), 7.31-7.23 (m, 4H), 7.21-7.05 (m, 8H), 6.77 (dd, J = 9.0, 3.1 Hz, 1H), 2.79 (t, J = 5.2, 5.0 Hz, 4H), 2.54 (tt, J = 11.0, 3.9 Hz, 1H), 2.17 (t, J = 5.2,

5.0 Hz, 4H), 2.01-1.99 (m, 1H), 1.93-1.77 (m, 4H), 1.57-1.51 (m, 2H), 1.34-1.26 (m, 2H). ¹H-NMR (300 MHz, CD₃OD, δ = ppm): δ 8.53 (d, J = 2.9 Hz, 1H), 8.30 (d, J = 9.4 Hz, 1H), 8.22 (dd, J = 9.5, 2.9 Hz, 1H), 8.06 (dt, J = 3.9, 2.6 Hz, 2H), 7.70-7.59 (m, 3H), 7.42 (d, J = 7.5 Hz, 2H), 7.32 (t, J = 7.6 Hz, 2H), 7.18 (t, J = 7.3 Hz, 1H), 4.33 (d, J = 14.2 Hz, 2H), 3.83 (d, J = 12.6 Hz, 2H), 3.73 (t, J = 13.2 Hz, 2H), 3.51 (dt, J = 7.4, 3.7 Hz, 1H), 3.37 (dd, J = 12.2, 2.4 Hz, 1H), 2.99 (dd, J = 8.7, 5.1 Hz, 1H), 2.38-2.29 (m, 2H), 2.12-1.86 (m, 6H). ¹³C-NMR (75 MHz, C₆D₆, δ = ppm): δ 156.9, 153.6, 147.9, 147.7, 136.4, 130.9, 129.3, 128.8, 127.3, 126.4, 123.6, 121.7, 113.4, 58.2, 49.5, 47.5, 44.2, 28.6. ¹³C-NMR (75 MHz, CD₃OD, δ = ppm): δ 153.5, 149.6, 144.5, 135.2, 131.1, 130.8, 129.6, 128.7, 128.3, 127.1, 125.0, 120.0, 66.3, 45.1, 39.6, 29.1, 25.0. HRMS m/z calculated for C₂₇H₃₂N₅: 426.2652 ([M+H]⁺); found: 426.2637.

1-((1S,4S)-4-phenylcyclohexyl)-4-(4-((E)-phenyldiazenyl)phenyl)piperazine, hydrochloride salt (9b). To a solution of **5b** (1.85 mmol, 560.0 mg) and **6** (1.68 mmol, 293.0 mg) in dry DCE (9.20 mL) was added Et₃N (3.60 mmol, 0.501 mL). The solution was stirred vigorously at r.t. for 6 h. NaBH(OAc)₃ (2.19 mmol, 464.0 mg) was added in small portions and the mixture was stirred for 2 days at r.t. The reaction was stopped by the addition of saturated solution of NaHCO₃ (50.0 mL). The mixture was extracted with CH₂Cl₂ (5 x 50.0 mL), the collected organic phase was washed with H₂O (2 x 50.0 mL), dried in Na₂SO₄, and the solvent was removed in vacuum. The crude was subjected to column chromatography (Hexanes 7:3 AcOEt + 0.5% Et₃N). The title compound was solubilized in a mixture a methanolic solution of HCl (1.25 M) and precipitated by the addition of dry diethylether to afford **9b** as a red powder (261.0 mg, 0.56 mmol, 34%). ¹H-NMR (300 MHz, C₆D₆, δ = ppm): δ 8.23 (d, J = 9.0 Hz, 2H), 8.16 (dd, J = 7.3, 1.3 Hz, 2H), 7.31-7.22 (m, 6H), 7.14-7.08 (m, 2H), 6.75 (d, J = 9.1 Hz, 2H), 2.93 (t, J = 10.1, 5.0 Hz, 4H), 2.55 (tt, J = 11.0, 3.8 Hz, 1H), 2.26 (t, J = 10.1, 5.0 Hz, 4H), 2.03 (t, J = 5.8, 2.9 Hz, 1H), 1.96-1.82 (m, 4H), 1.55 (dd, J = 12.9, 3.6 Hz, 1H), 1.31 (t, J = 13.1 Hz, 2H). ¹³C-NMR (75 MHz, C₆D₆, δ = ppm): δ 153.9, 153.6, 147.7, 146.1, 130.0, 129.3, 128.8, 128.3, 127.3, 126.4, 125.3, 123.0, 114.7, 58.3, 49.8, 48.1, 44.3, 28.7. HRMS m/z calculated for C₂₈H₃₃N₄: 425.2700 ([M+H]⁺); found: 425.2698.

1-((1S,4S)-4-phenylcyclohexyl)-4-(4-((E)-pyridin-3-yl)diazenyl)phenyl)piperazine, double hydrochloride salt (9c). To a solution of **5c** (2.20 mmol, 750.0 mg) and **6** (2.00 mmol, 348.0 mg) in dry DCE (10.00 mL) was added Et₃N (4.80 mmol, 0.669 mL). The solution was stirred vigorously at r.t. for 3 h. NaBH(OAc)₃ (2.60 mmol, 551.0 mg) was added in small portions and the mixture was stirred for 2 days

at r.t. The reaction was quenched by the addition of saturated solution of NaHCO_3 (50.0 mL). The mixture was extracted with CH_2Cl_2 (5 x 50.0 mL). The collected organic phase was washed with H_2O (2 x 50.0 mL), dried over Na_2SO_4 , and the solvents were evaporated under vacuum. The crude was subjected to column chromatography (Hexanes 7:3 AcOEt + 0.5% Et_3N to Hexanes 3:7 AcOEt + 0.5% Et_3N). The title compound was solubilized in a mixture a methanol solution of HCl (1.25 M) and precipitated by the addition of dry diethylether to afford **9c** as a red powder (397.0 mg, 0.80 mmol, 40%). ^1H -NMR (300 MHz, C_6D_6 , δ = ppm): δ 9.57 (d, J = 2.1 Hz, 1H), 8.50 (dd, J = 4.7, 1.6 Hz, 1H), 8.15 (dt, J = 6.9, 3.1, 2.0 Hz, 2H), 8.02 (ddd, J = 8.1, 2.3, 1.7 Hz, 1H), 7.31-7.24 (m, 5H), 6.77 (ddd, J = 8.1, 4.7, 0.6 Hz, 1H), 6.72 (dt, J = 9.2, 3.0, 1.9 Hz, 2H) 2.94 (t, J = 10.2, 5.0 Hz, 4H), 2.55 (tt, J = 11.0, 3.9 Hz, 1H), 2.25 (d, J = 10.2, 5.1 Hz, 4H), 2.03 (t, J = 5.6, 2.8 Hz, 1H), 1.94 (ddd, J = 9.6, 2.8, 1.6 Hz, 2H), 1.85-1.81 (m, 2H), 1.57-1.53 (m, 2H), 1.36-1.28 (m, 2H). ^{13}C -NMR (75 MHz, C_6D_6 , δ = ppm): δ 153.9, 151.1, 148.8, 147.7, 147.6, 145.9, 128.8, 127.3, 126.4, 126.2, 125.5, 123.8, 114.5, 58.4, 49.8, 47.9, 44.2, 28.7. HRMS m/z calculated for $\text{C}_{27}\text{H}_{32}\text{N}_5$: 426.2652 ($[\text{M}+\text{H}]^+$); found: 426.2652.

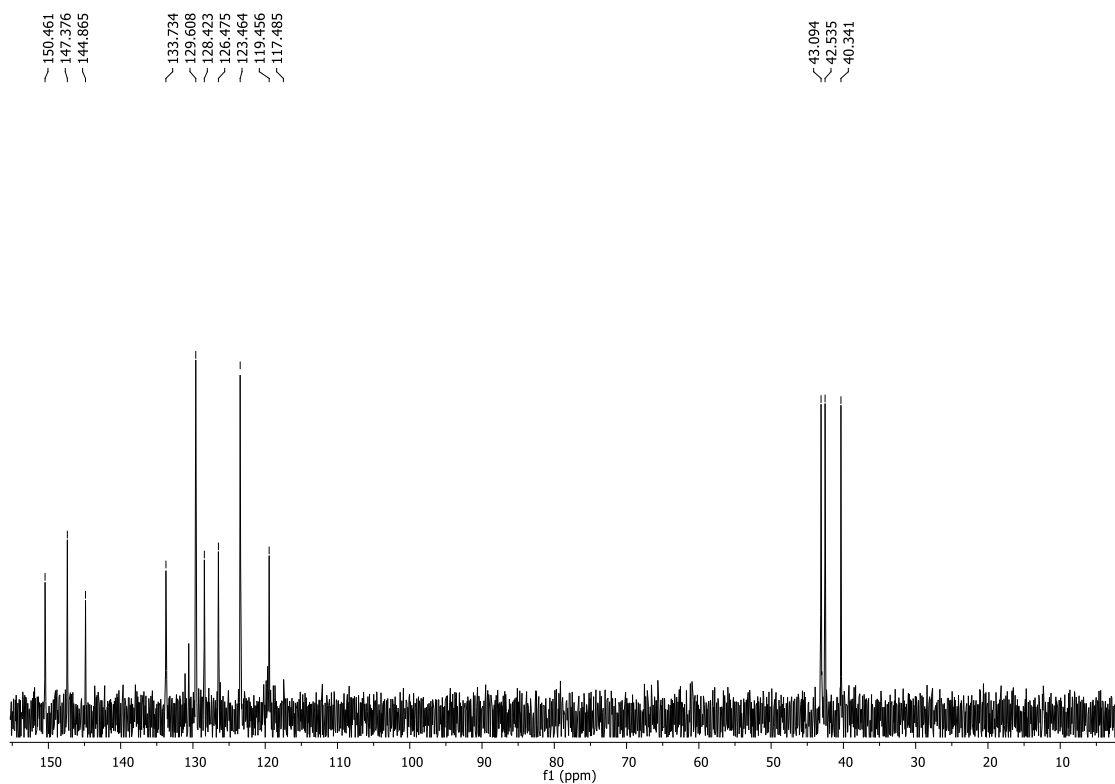
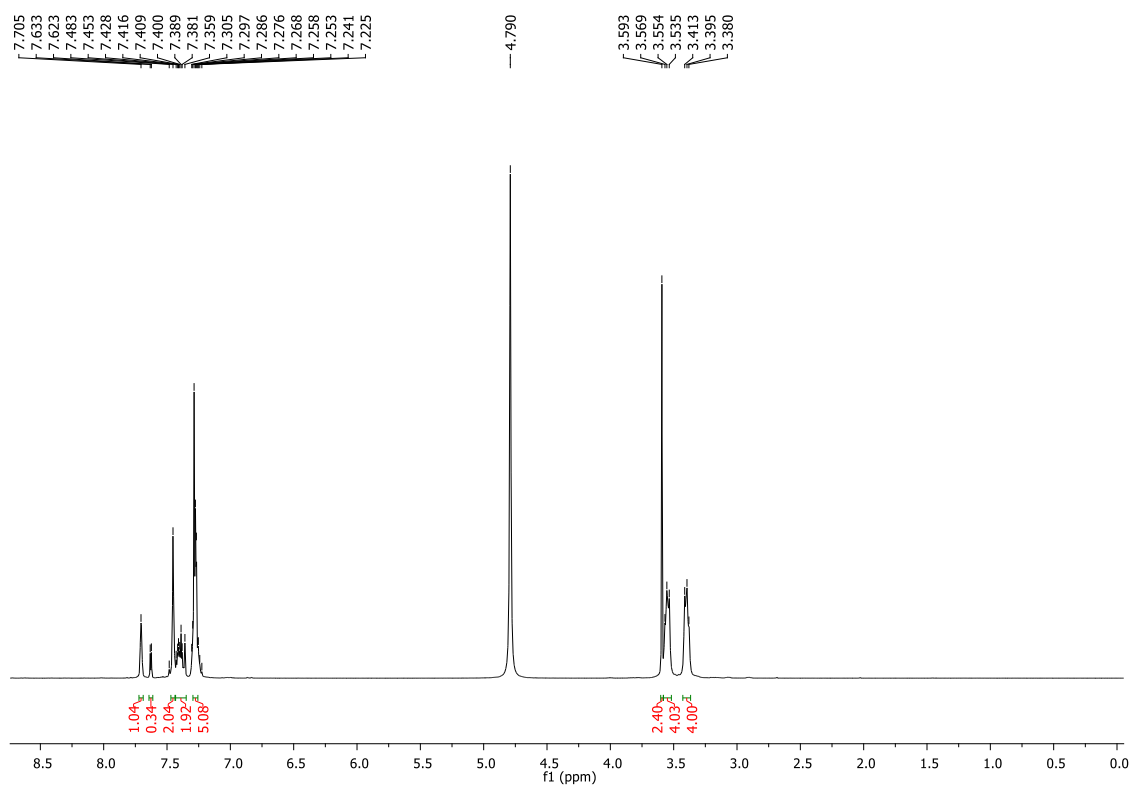
1-((1S,4S)-4-phenylcyclohexyl)-4-((6-((E)-phenyldiazenyl)pyridin-3-yl)methyl)piperazine, triple hydrochloride salt (9d). A solution of **5d** (0.67 mmol, 143.6 mg) and Et_3N (2.14 mmol, 0.298 mL) in THF (6.4 mL) was cooled to 0 °C, under stirring. MsCl (1.34 mmol, 0.104 mL) was added dropwise over a period of 5 minutes. After addition, the mixture stirred for additional 10 minutes at 0 °C. the ice bath was removed and the mixture was allowed to warm to r.t. and stirred for 2h. After complete consumption of the starting materials (monitored by TLC) the reaction mixture was filtered through Celite, washed several times with THF, and concentrated in vacuum. **Ms-5d** was obtained as an orange oil (195.2 mg) and was used in the next step without further purification. Then, **Ms-5d** (0.67 mmol, 195.2 mg), **8** (0.80 mmol, 224.7 mg), K_2CO_3 (2.00 mmol, 276.4 mg), and NaI (0.07 mmol, 10.5 mg) were suspended in DMF (2.5 mL) and stirred at 60 °C for 2 h. After completion (monitored by TLC), the reaction was partially concentrated under vacuum and diluted with AcOEt (20.0 mL). The solution was filtered over a pad of silica (Hexanes 5:5 AcOEt), and the solvents were evaporated. H_2O (25.0 mL) was added to the crude and the suspension was extracted with AcOEt (3 x 25.0 mL). The collected organic phase was extracted with an aqueous solution of HCl (1.0 M, 2 x 25.0 mL). The resulting aqueous phase was neutralized with saturated solution of NaHCO_3 and extracted with AcOEt (2 x 25.0 mL). The combined organic phases was dried over Na_2SO_4 and evaporated under vacuum. The crude was column

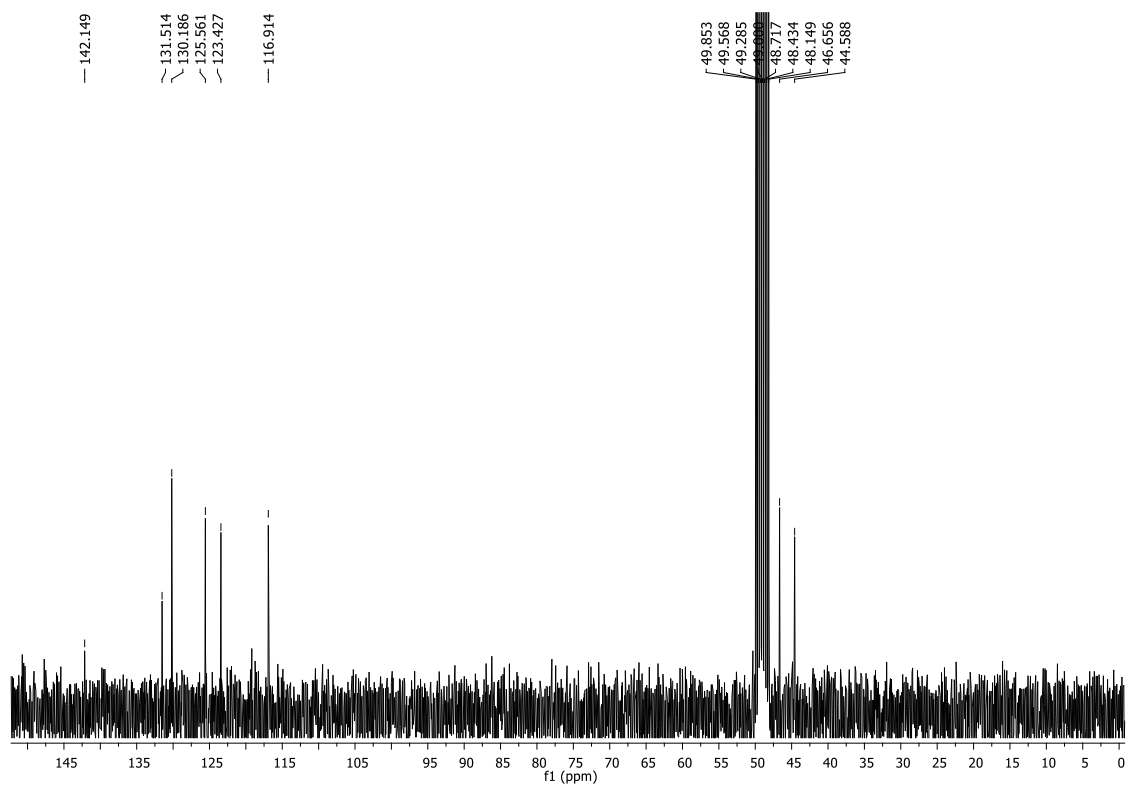
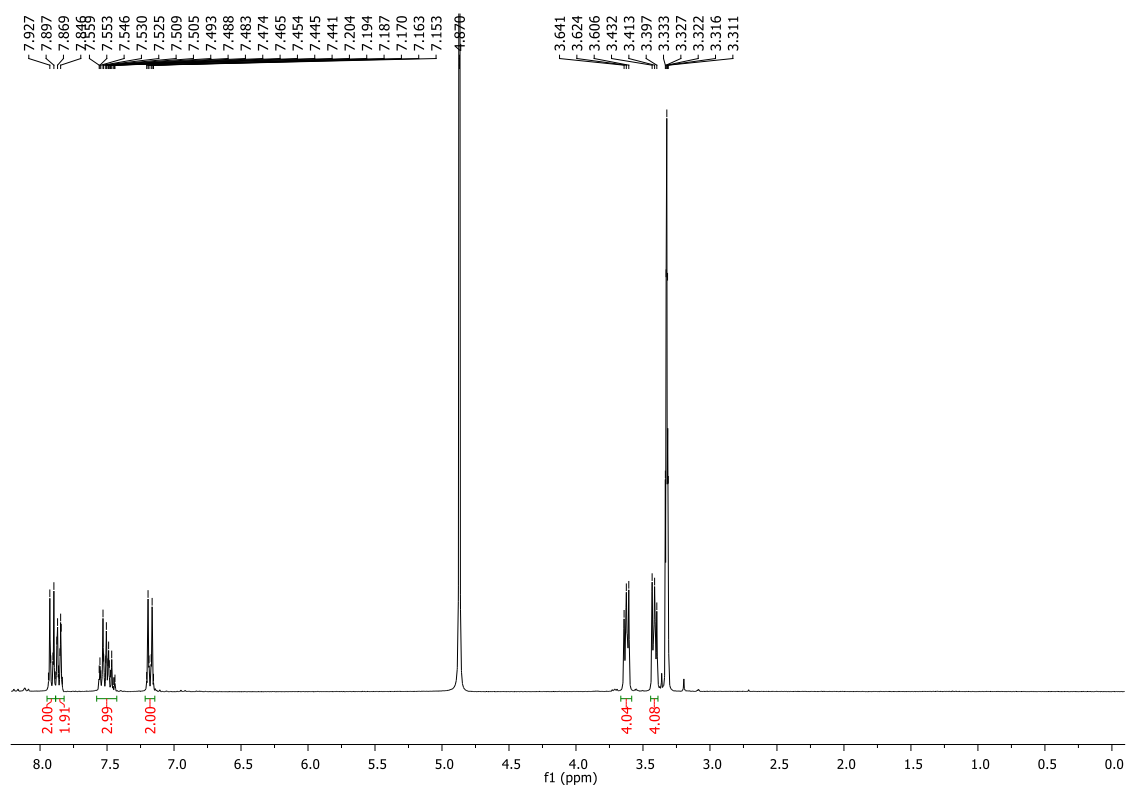
chromatographed (Hexanes 5:5 AcOEt + 0.5% Et₃N). The title compound was solubilized in a mixture a methanolic solution of HCl (1.25 M) to afford **9d** as a red powder (75.0 mg, 0.14 mmol, 20%). ¹H-NMR (300 MHz, C₆D₆, δ = ppm): δ 8.74 (d, *J* = 1.8 Hz, 1H), 8.12 (dd, *J* = 6.9, 1.5 Hz, 2H), 7.69 (d, *J* = 8.2 Hz, 1H), 7.50 (dd, *J* = 8.2, 2.2 Hz, 1H), 7.30-7.20 (m, 4H), 7.13-7.04 (m, 4H), 3.13 (s, 2H), 2.53 (tt, *J* = 11.0, 7.9, 3.8 Hz, 1H), 2.30 (d, *J* = 15.0 Hz, 8H), 2.09 (t, *J* = 2.5 Hz, 1H), 1.95 (ddd, *J* = 29.4, 15.4, 2.0 Hz, 4H), 1.54 (dd, *J* = 12.8, 3.6 Hz, 2H), 1.32 (t, *J* = 13.0 Hz, 2H). ¹H-NMR (300 MHz, DMSO-*d*₆, δ = ppm): δ 11.54 (bs, 1H), 8.97 (d, *J* = 1.7 Hz, 1H), 8.38 (dd, *J* = 8.3, 2.1 Hz, 1H), 7.97 (dt, *J* = 7.6, 3.4 Hz, 2H), 7.80 (d, *J* = 8.2 Hz, 1H), 7.67 (dd, *J* = 6.6, 3.6 Hz, 3H), 7.43 (d, *J* = 7.4 Hz, 2H), 7.28 (t, *J* = 7.5 Hz, 2H), 7.17 (t, *J* = 7.3 Hz, 1H), 5.56 (s, 4H), 4.59 (s, 2H), 3.80 (t, *J* = 15.0 Hz, 4H), 3.65-3.34 (m, 5H), 2.80-2.74 (m, 1H), 2.25-2.18 (m, 2H), 1.94-1.81 (m, 2H), 1.67 (d, *J* = 10.7 Hz, 1H). ¹³C-NMR (75 MHz, C₆D₆, δ = ppm): δ 163.8, 153.2, 150.4, 147.8, 138.3, 136.4, 131.9, 129.4, 128.7, 127.3, 126.3, 123.8, 112.6, 59.9, 58.7, 54.0, 50.3, 44.3, 28.8, 28.7. HRMS *m/z* calculated for C₂₈H₃₄N₅: 440.2809 ([M+H]⁺); found: 440.2796.

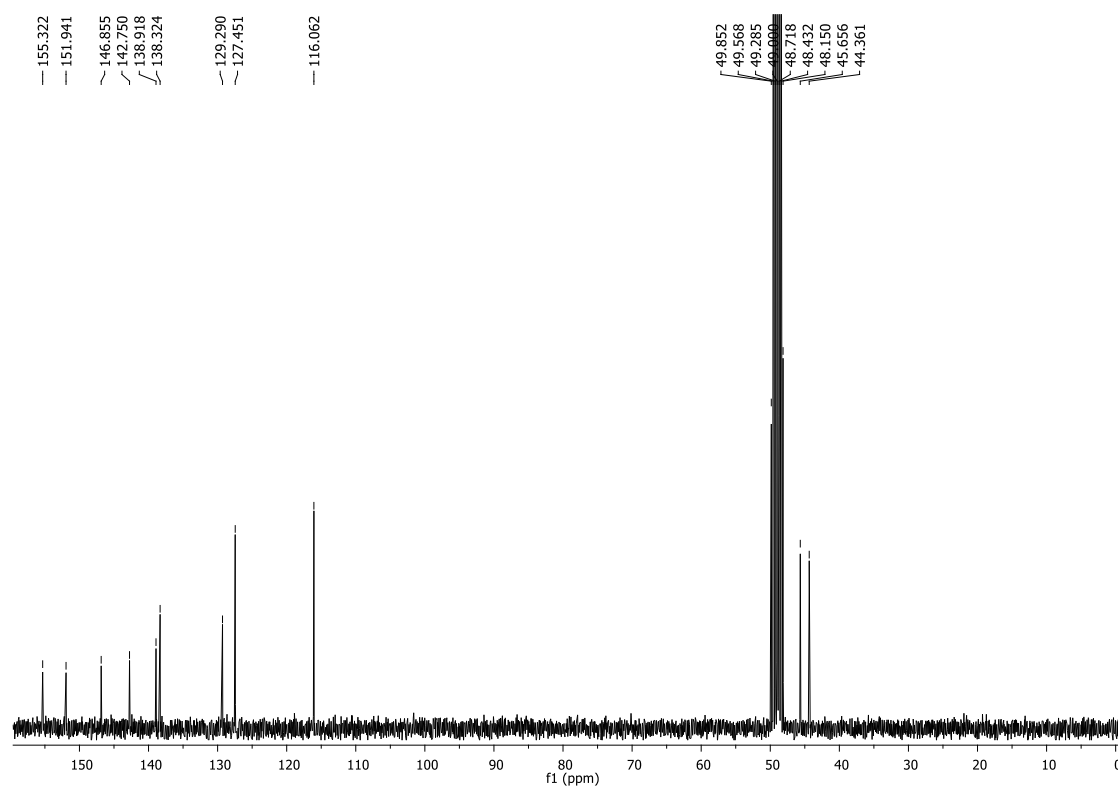
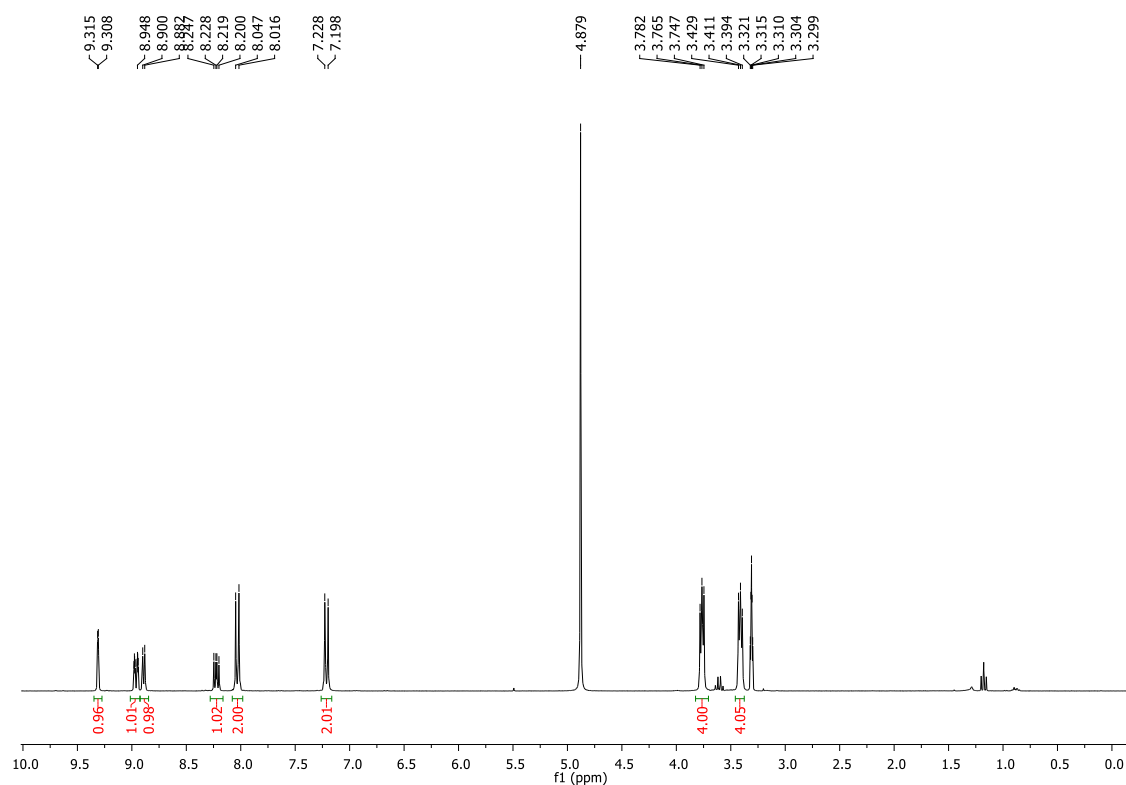
1-((1S,4S)-4-phenylcyclohexyl)-4-(4-((E)-phenyldiazenyl)benzyl)piperazine, double hydrochloride salt (9e). K₂CO₃ (6.00 mmol, 829.0 mg) was added to a stirring solution of **5e** (1.50 mmol, 413.0 mg) and **8** (1.50 mmol, 367.0 mg) in DMF (10.00 mL). The solution was stirred vigorously at r.t. for 24 h. The reaction was quenched by the addition of H₂O (50.0 mL). The precipitates were collected and washed with H₂O. After removal of the residual solvent under vacuum, the crude was subjected to column chromatography (Hexanes 5:5 AcOEt + 0.5% Et₃N). The title compound was solubilized in a mixture a methanol solution of HCl (1.25 M) and precipitated by the addition of dry diethylether to afford **9e** as a orange powder (350.0 mg, 0.68 mmol, 46%). ¹H-NMR (300 MHz, C₆D₆, δ = ppm): δ 8.12-8.05 (m, 4H), 7.43 (d, *J* = 8.4 Hz, 2H), 7.30-7.19 (m, 6H), 7.10 (ddt, *J* = 7.2, 4.6, 1.4 Hz, 2H), 3.33 (s, 2H), 2.53 (tt, *J* = 11.1, 7.8, 3.8 Hz, 1H), 2.40 (s, 8H), 2.11 (t, *J* = 5.9, 2.9 Hz, 1H), 2.05-1.86 (m, 4H), 1.56-1.51 (m, 2H), 1.36-1.26 (m, 2H). ¹³C-NMR (75 MHz, C₆D₆, δ = ppm): δ 153.4, 152.6, 147.9, 142.9, 131.0, 129.9, 129.3, 128.7, 127.4, 126.2, 123.4, 123.3, 62.9, 58.7, 54.1, 50.4, 44.4, 28.9, 28.7. HRMS *m/z* calculated for C₂₉H₃₅N₄: 439.2856 ([M+H]⁺); found: 439.2844.

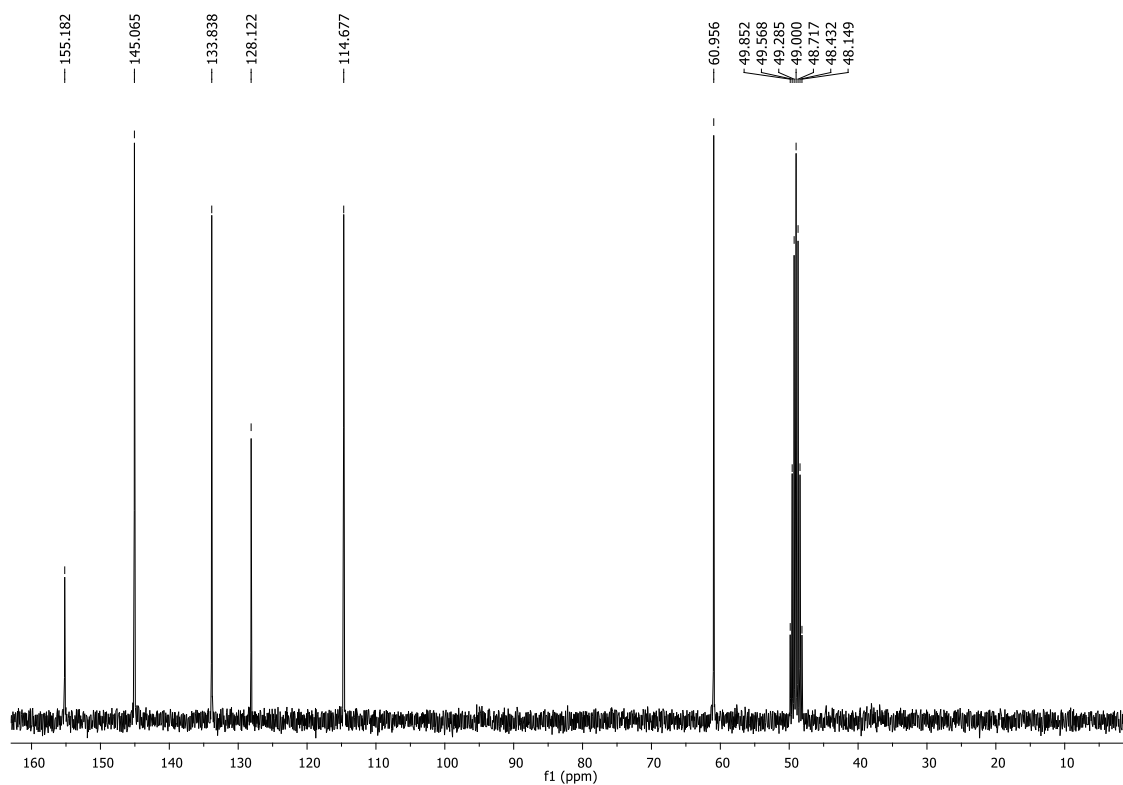
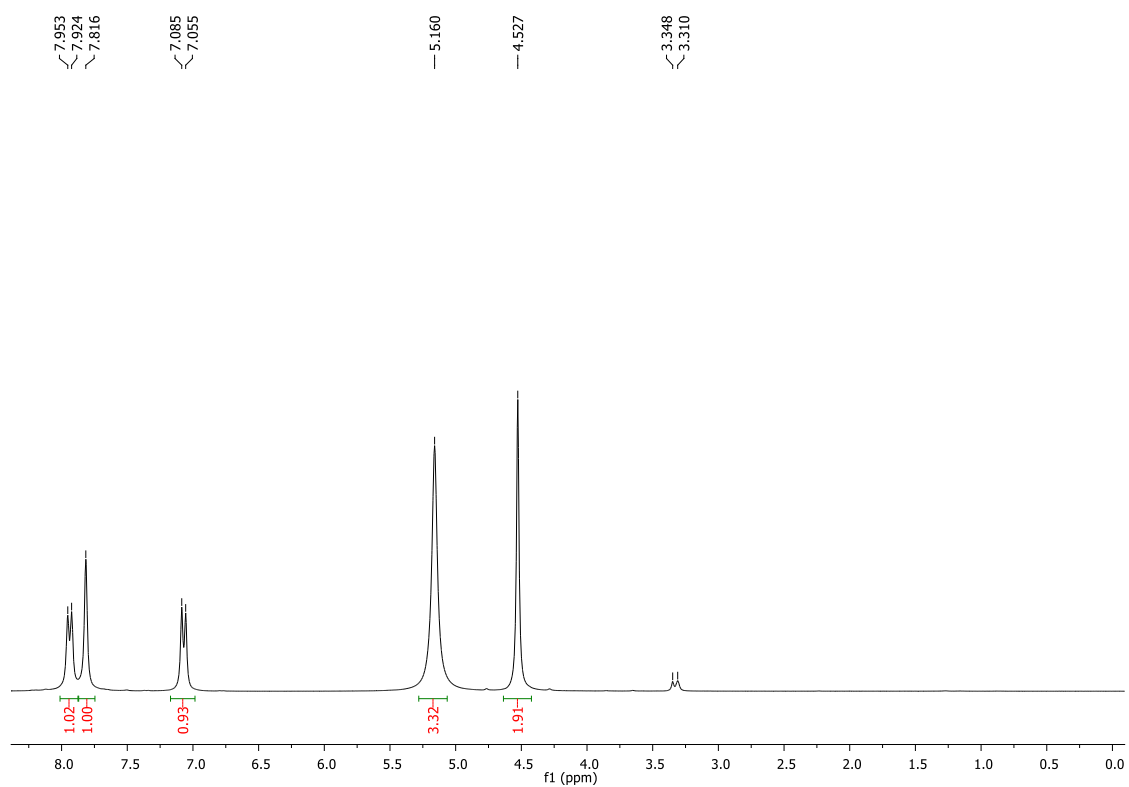
3. Copy of ^1H and ^{13}C NMR spectra

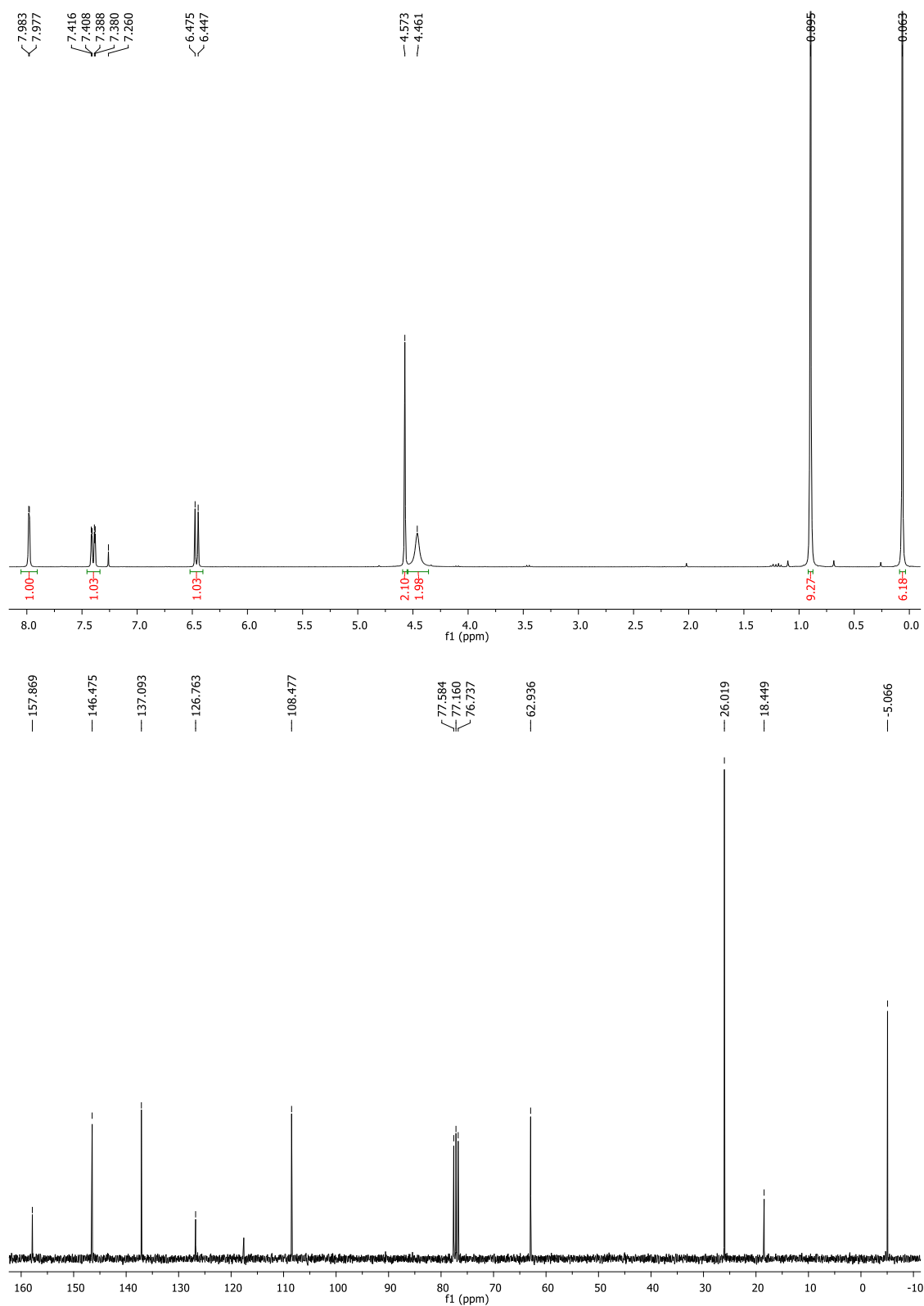
(E)-1-(6-(phenyldiazenyl)pyridin-3-yl)piperazine, double hydrochloride salt (5a)

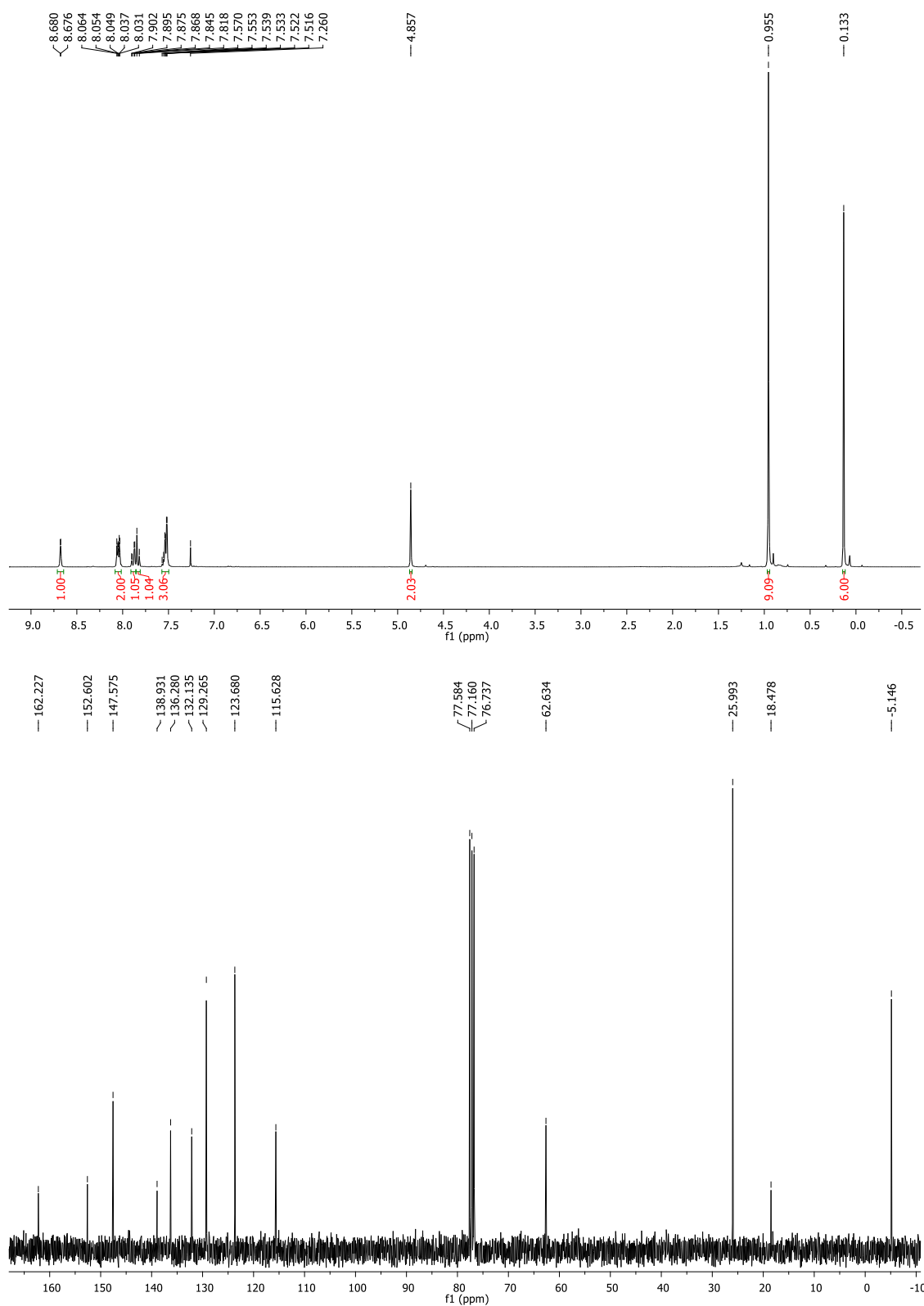


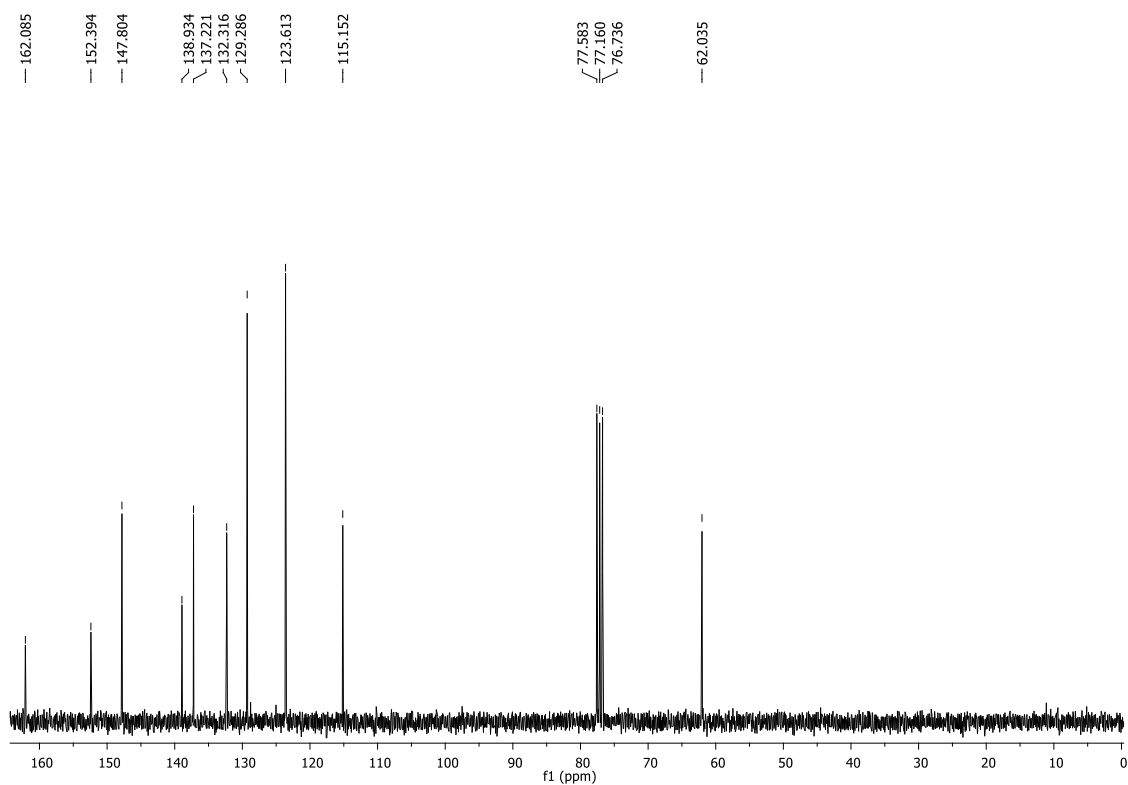
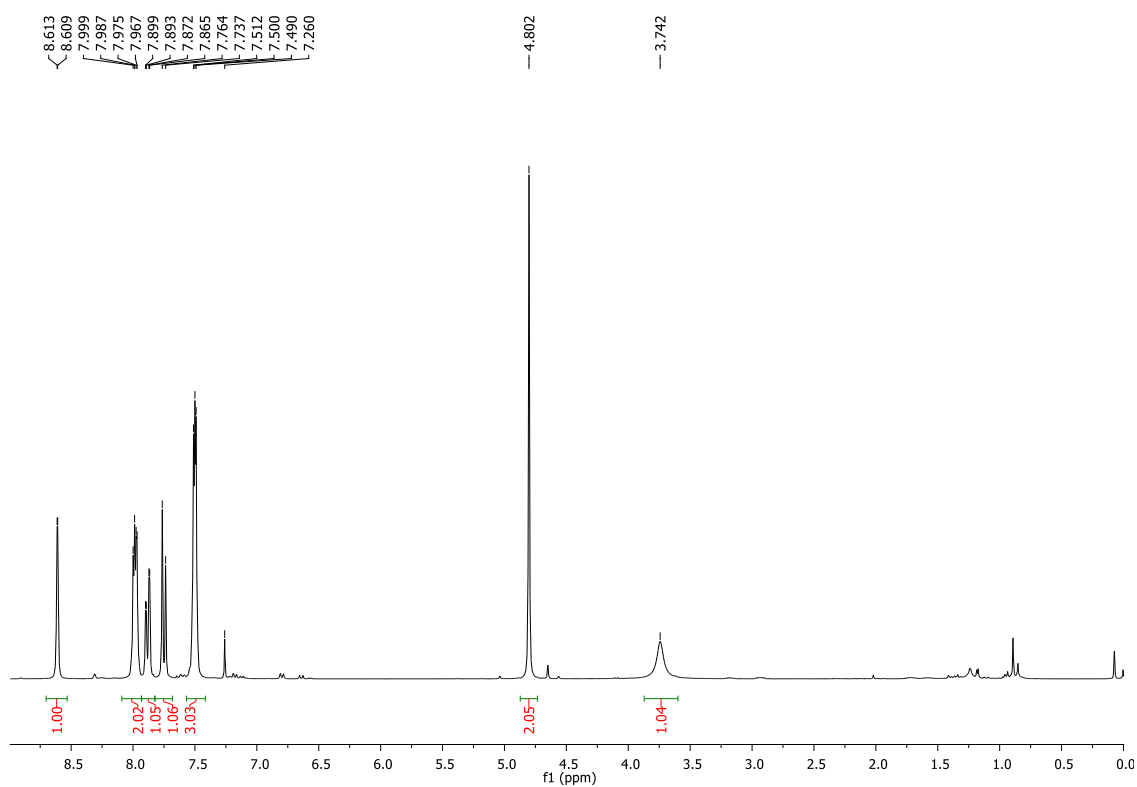
(E)-1-(4-(phenyldiazenyl)phenyl)piperazine, hydrochloride salt (5b)

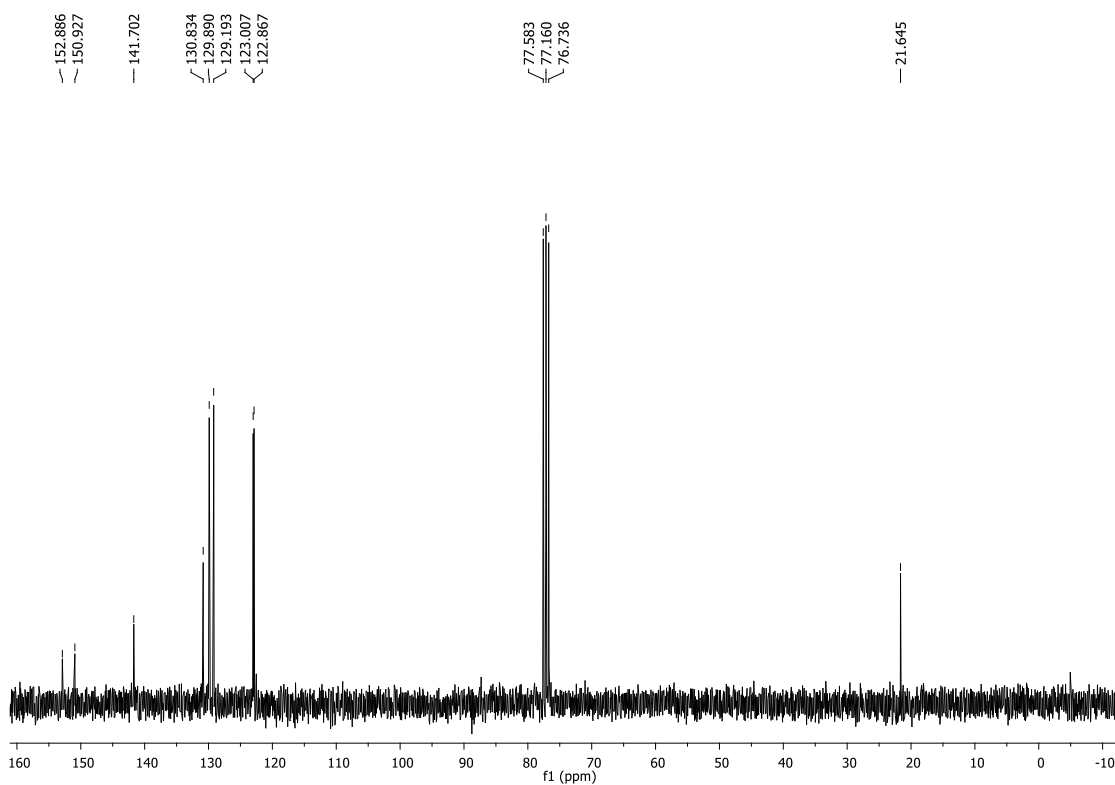
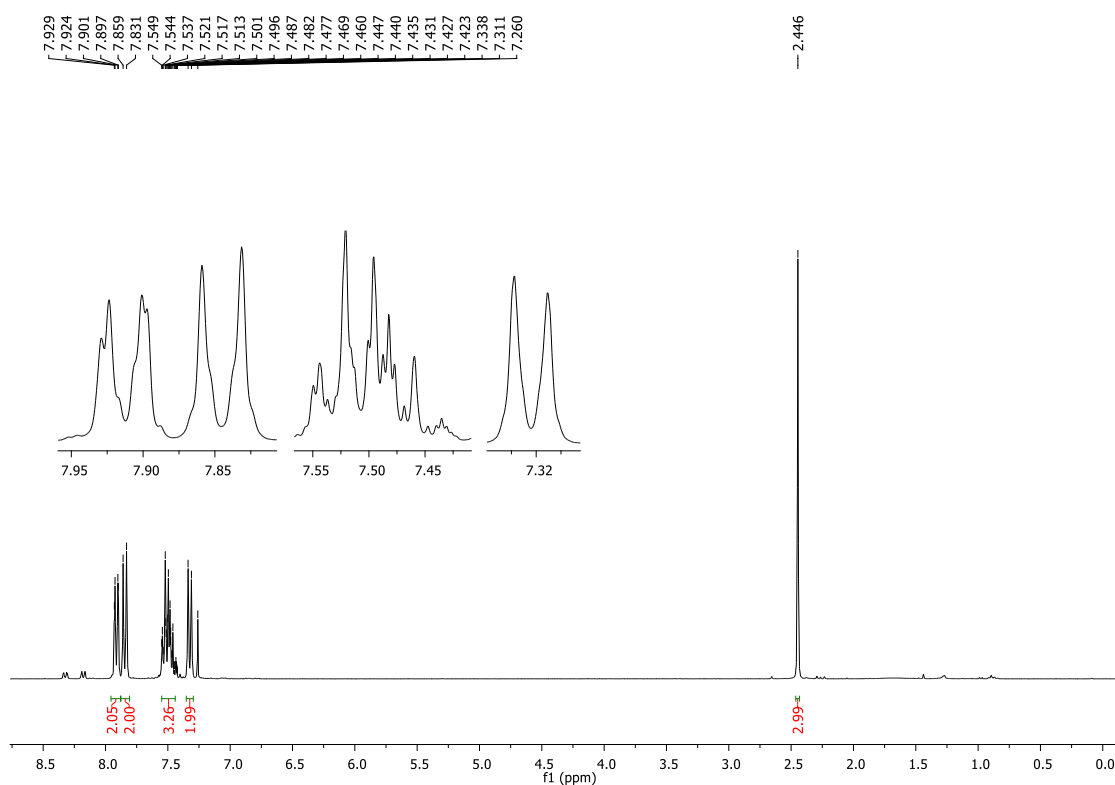
(E)-(4-piperazin-1-ylphenyl)-(3-pyridyl)diazene, double hydrochloride salt (5c)

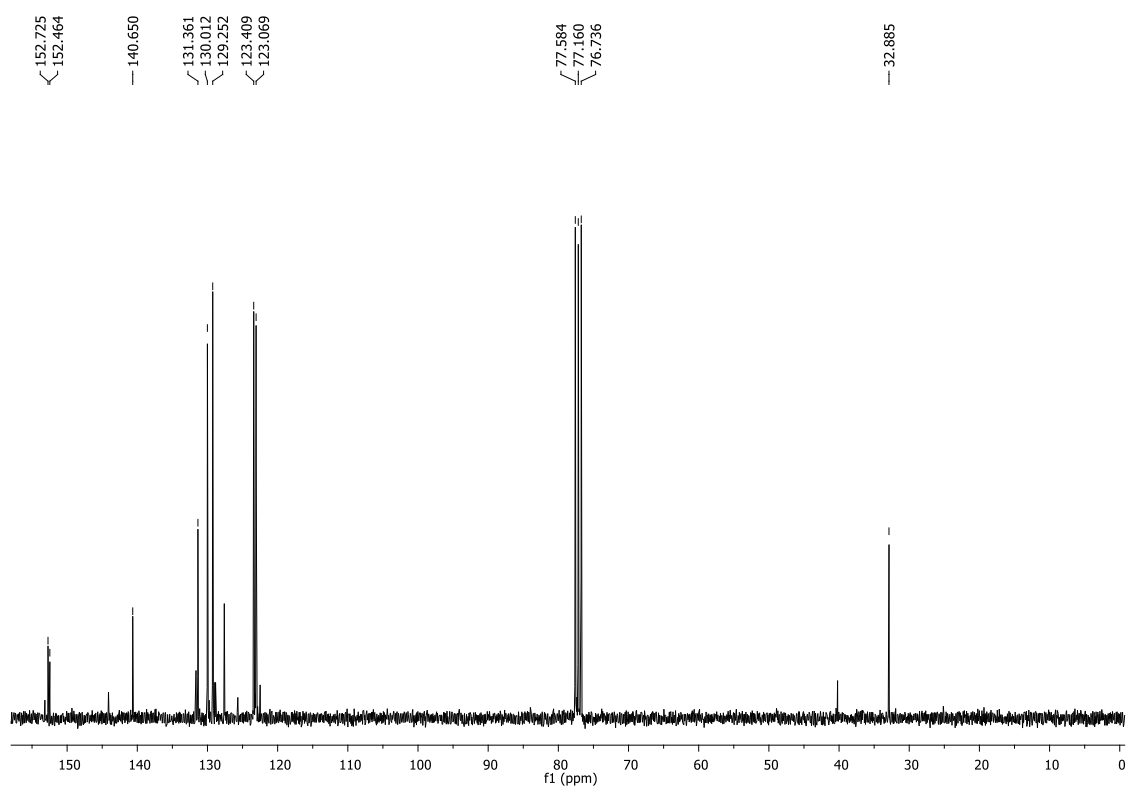
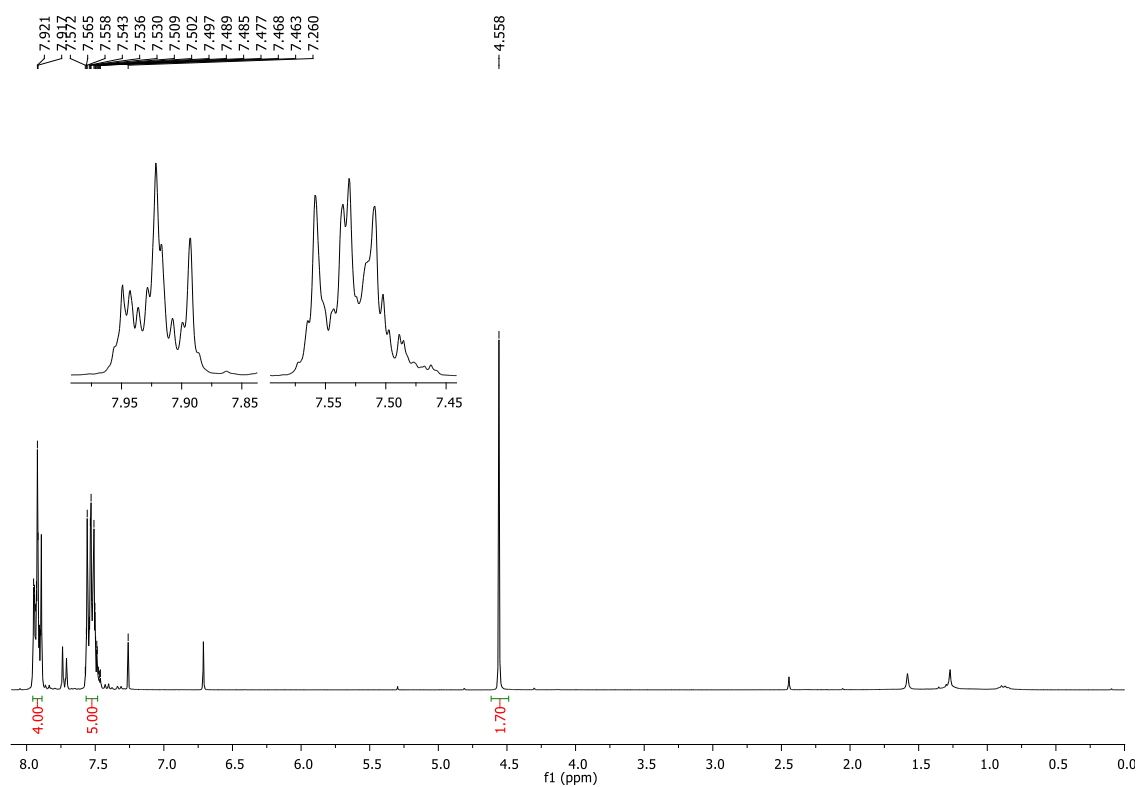
(6-aminopyridin-3-yl)methanol (4d)

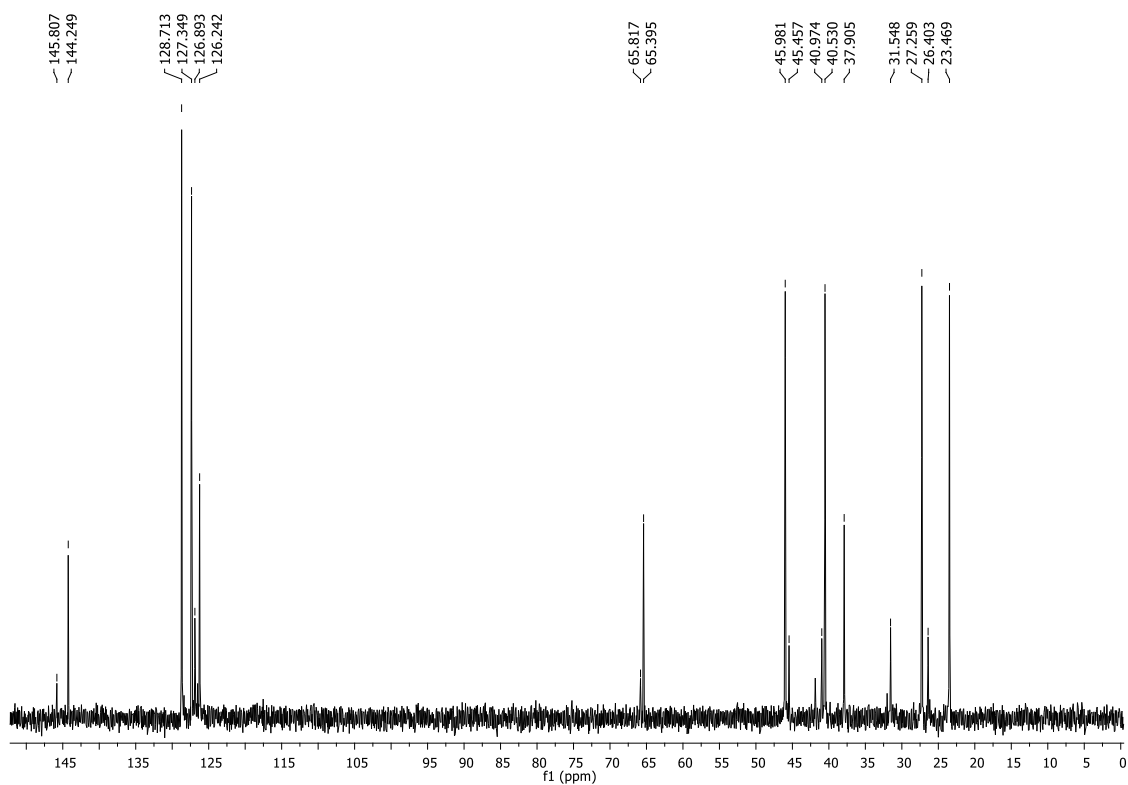
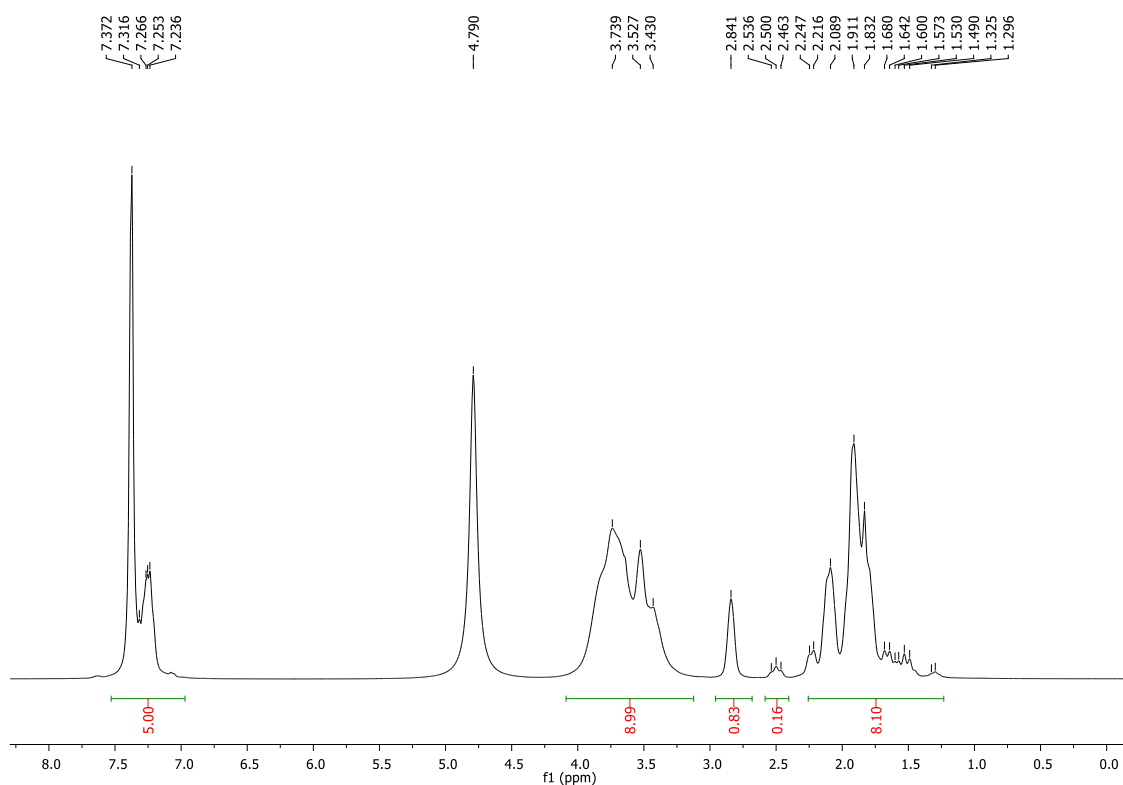
5-(((tert-butyldimethylsilyl)oxy)methyl)pyridin-2-amine (p-4d)

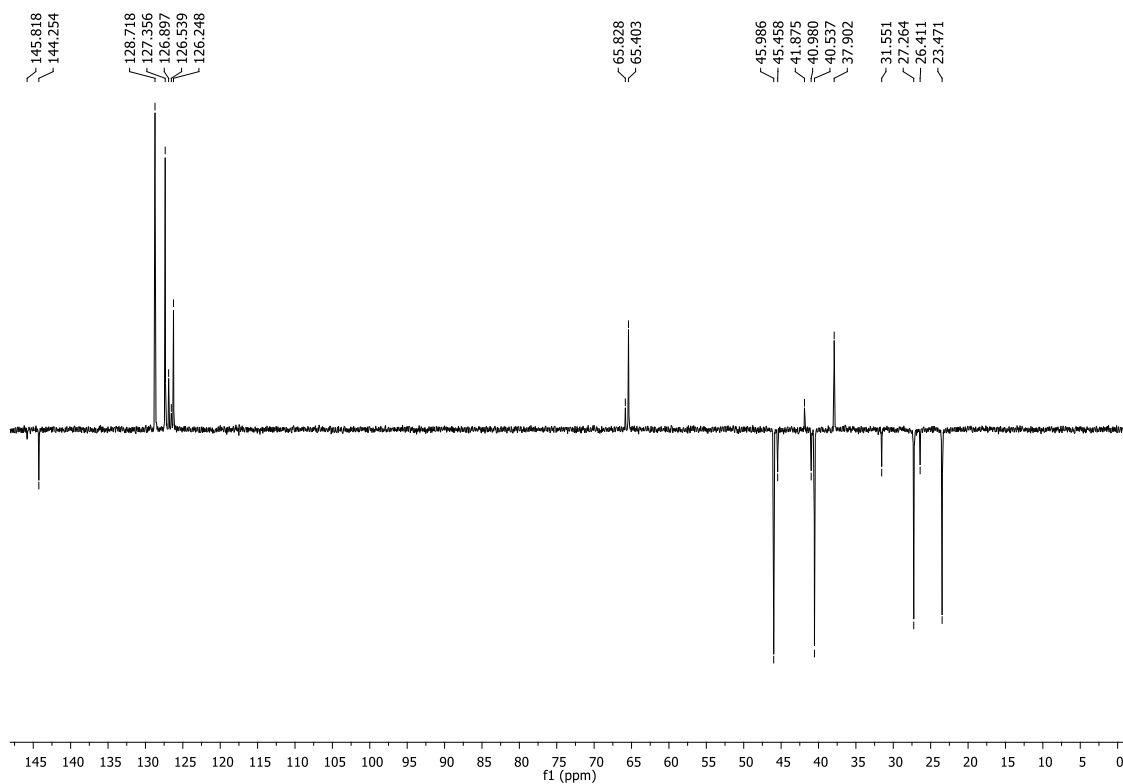
(E)-5-(((tert-butyldimethylsilyl)oxy)methyl)-2-(phenyldiazenyl)pyridine (p-5d)

(E)-(6-(phenyldiazenyl)pyridin-3-yl)methanol (5d)

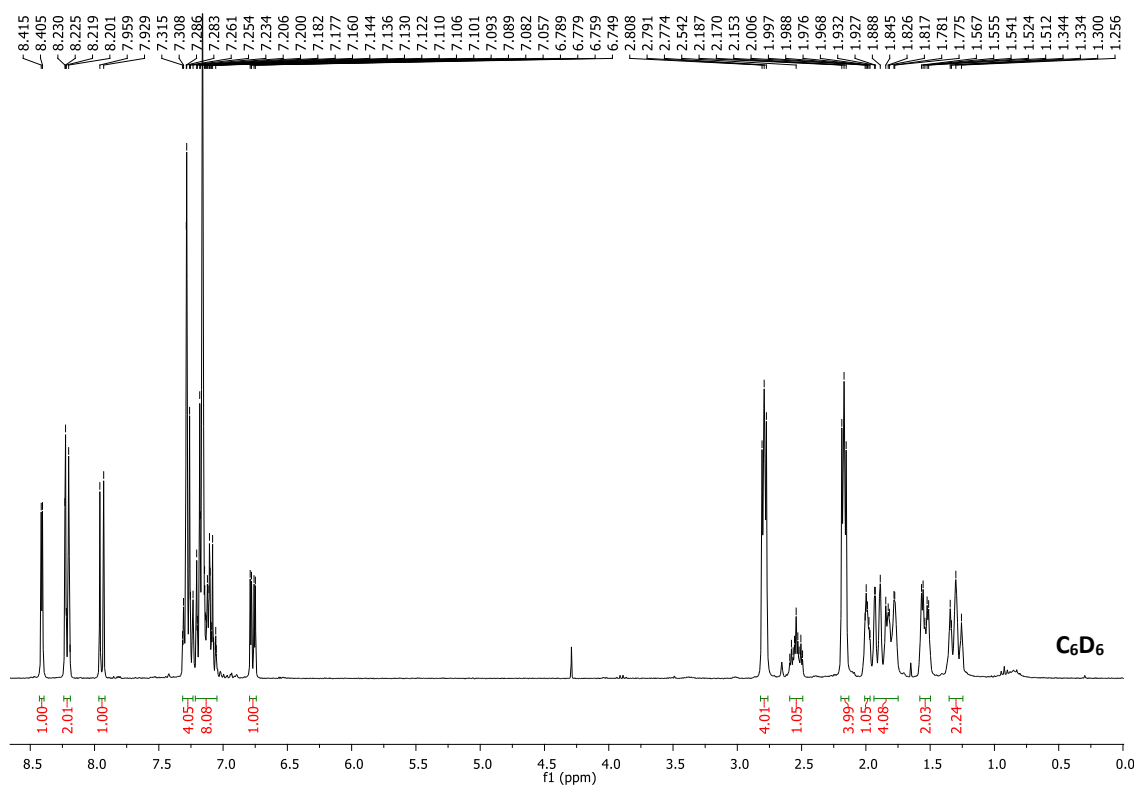
(E)-1-phenyl-2-(p-tolyl)diazene (4e)

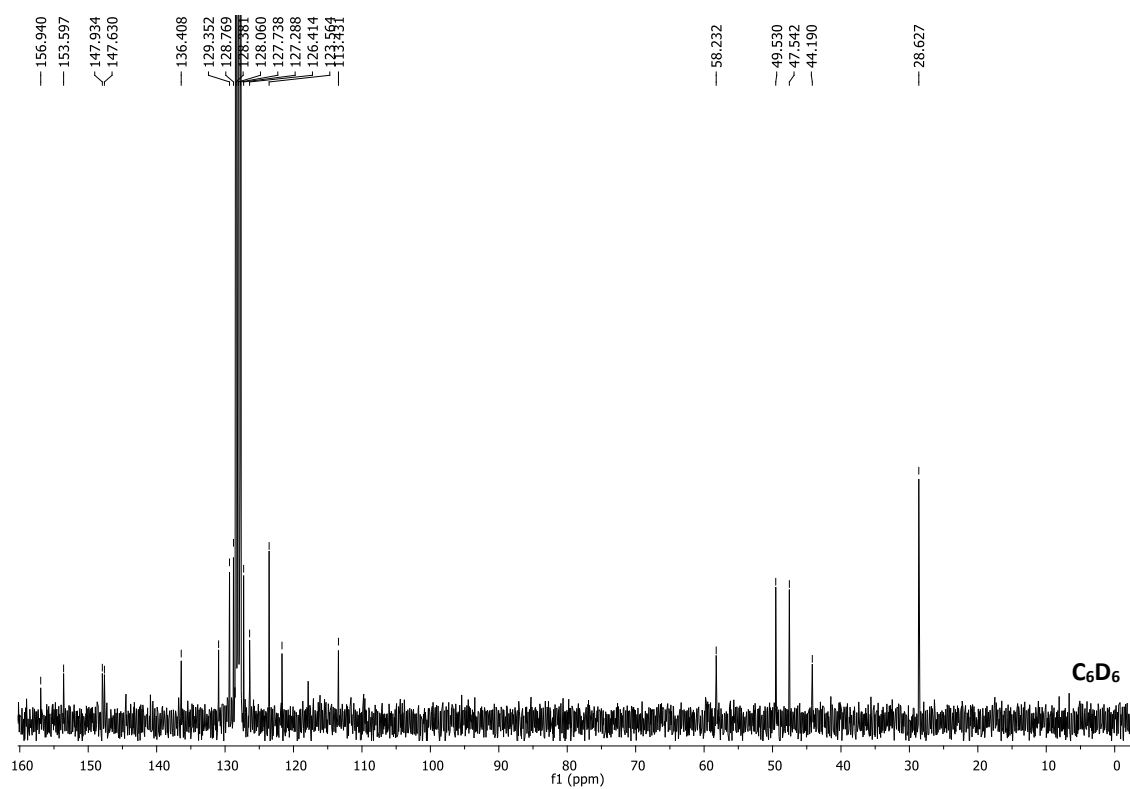
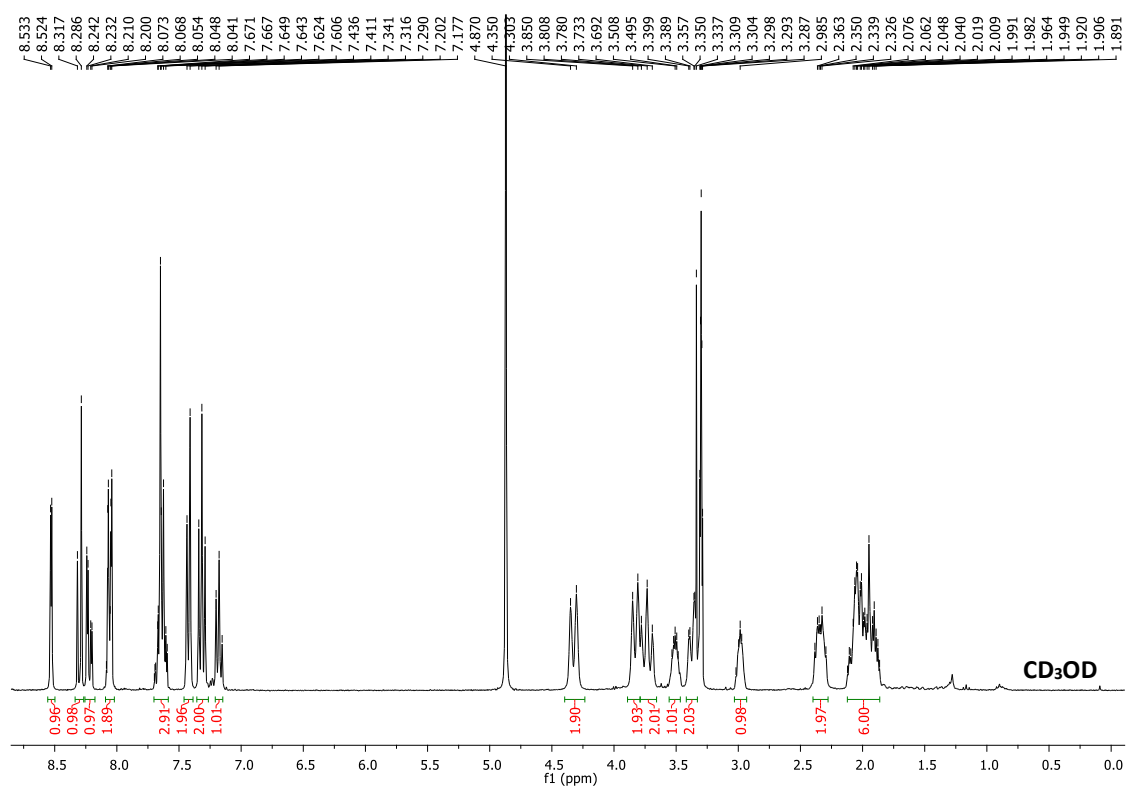
(E)-1-(4-(bromomethyl)phenyl)-2-phenyldiazene (5e)

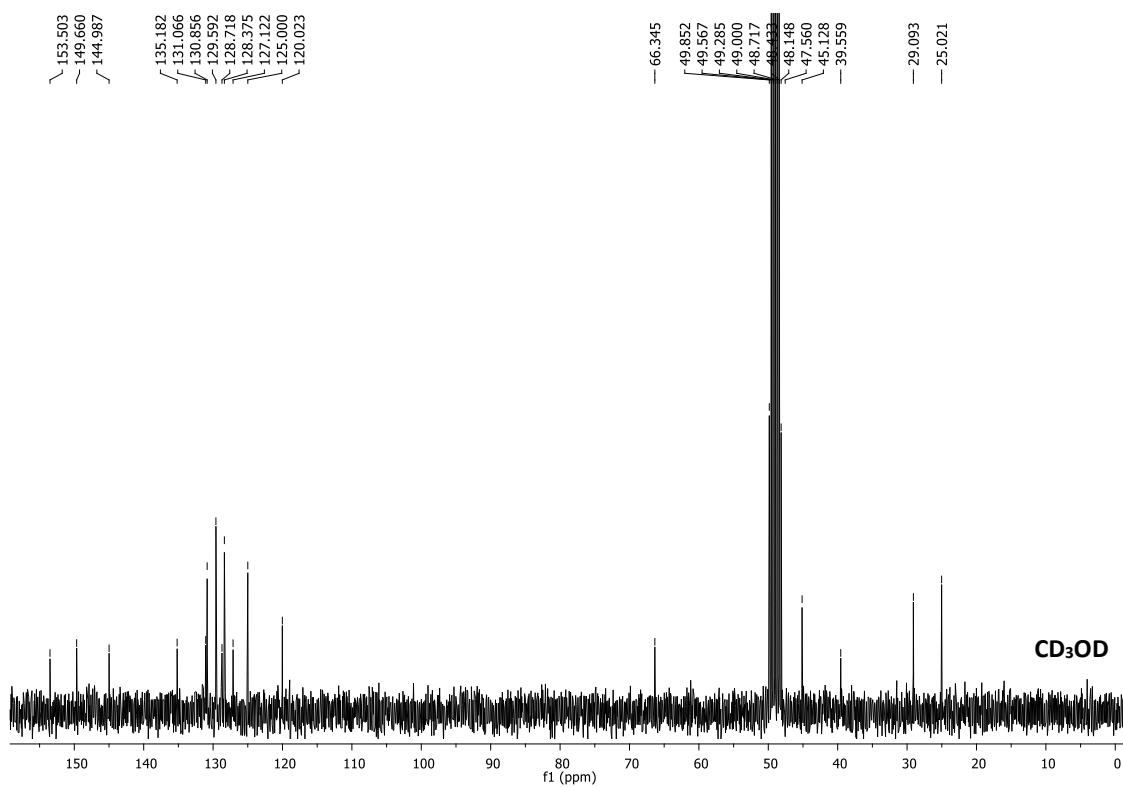
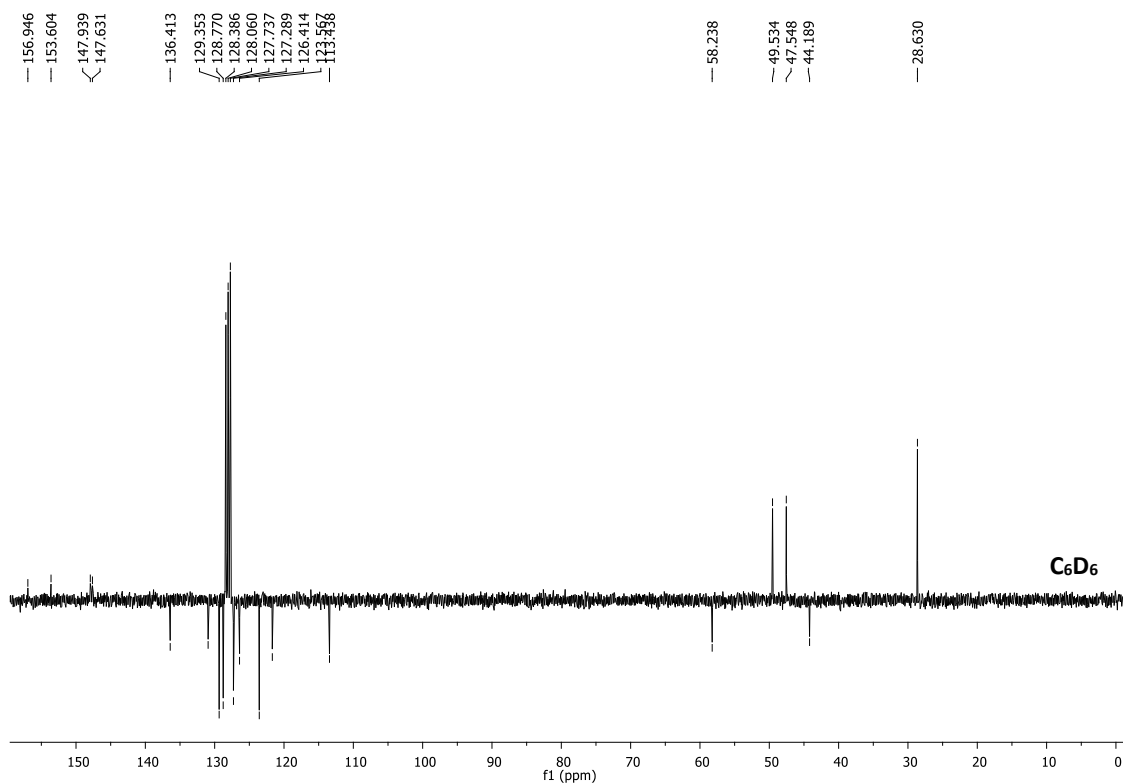
1-(4-phenylcyclohexyl)piperazine, double hydrochloride salt (8)

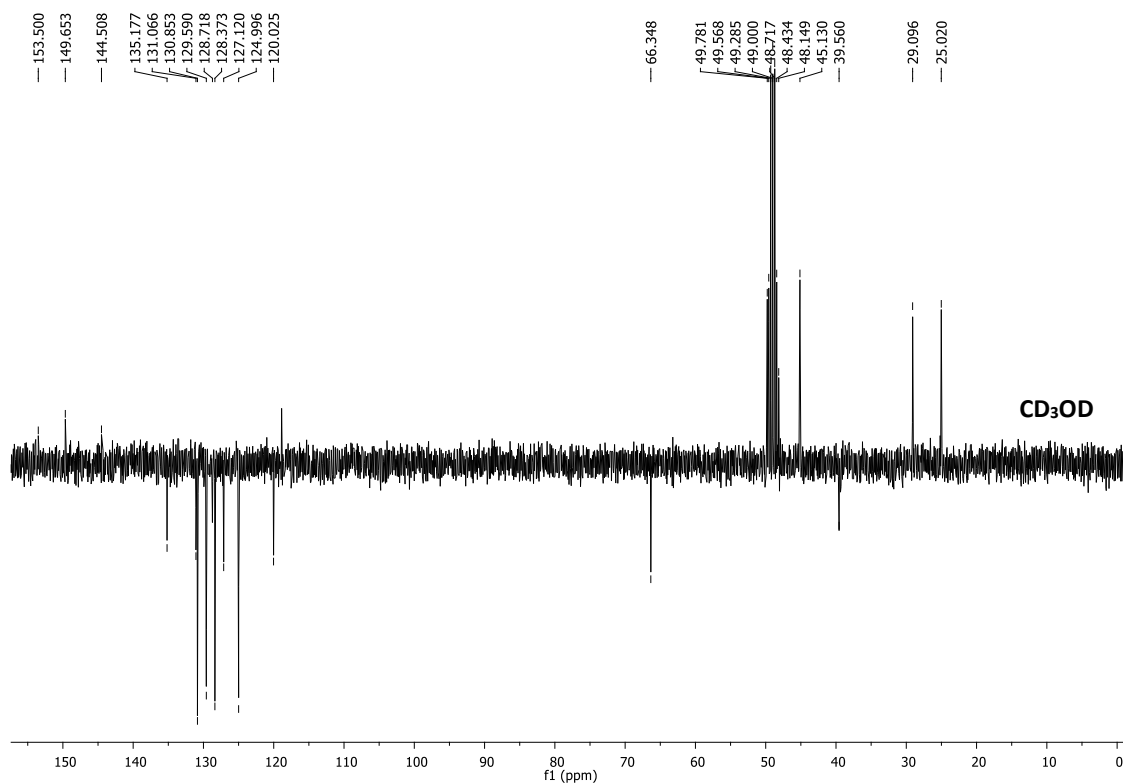


1-((1*S*,4*S*)-4-phenylcyclohexyl)-4-(6-((*E*)-phenyldiazenyl)pyridin-3-yl)piperazine, double hydrochloride salt (9a)

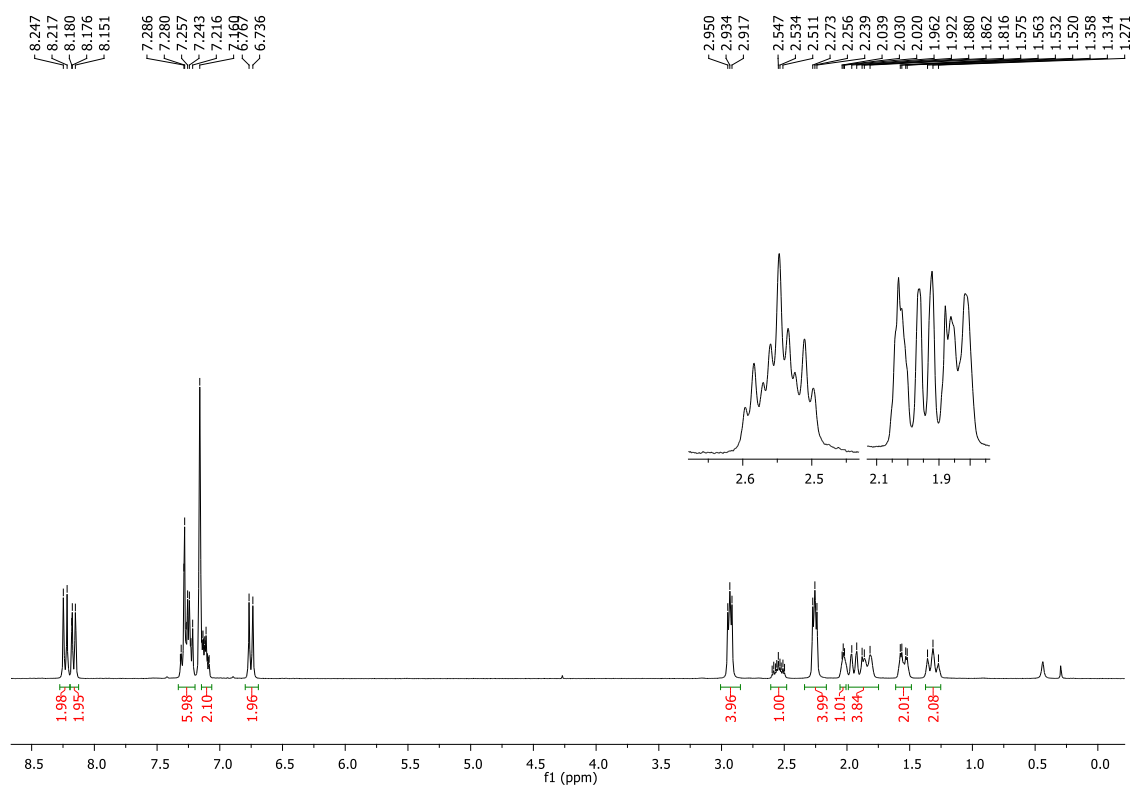


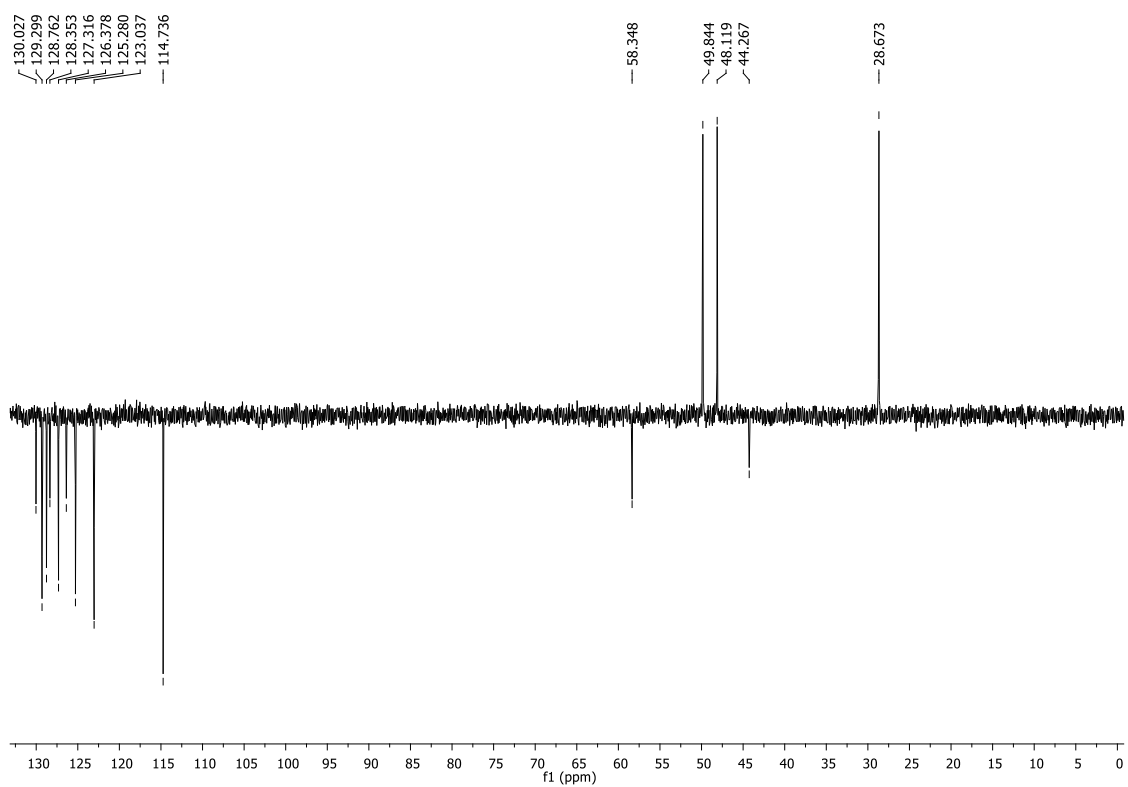
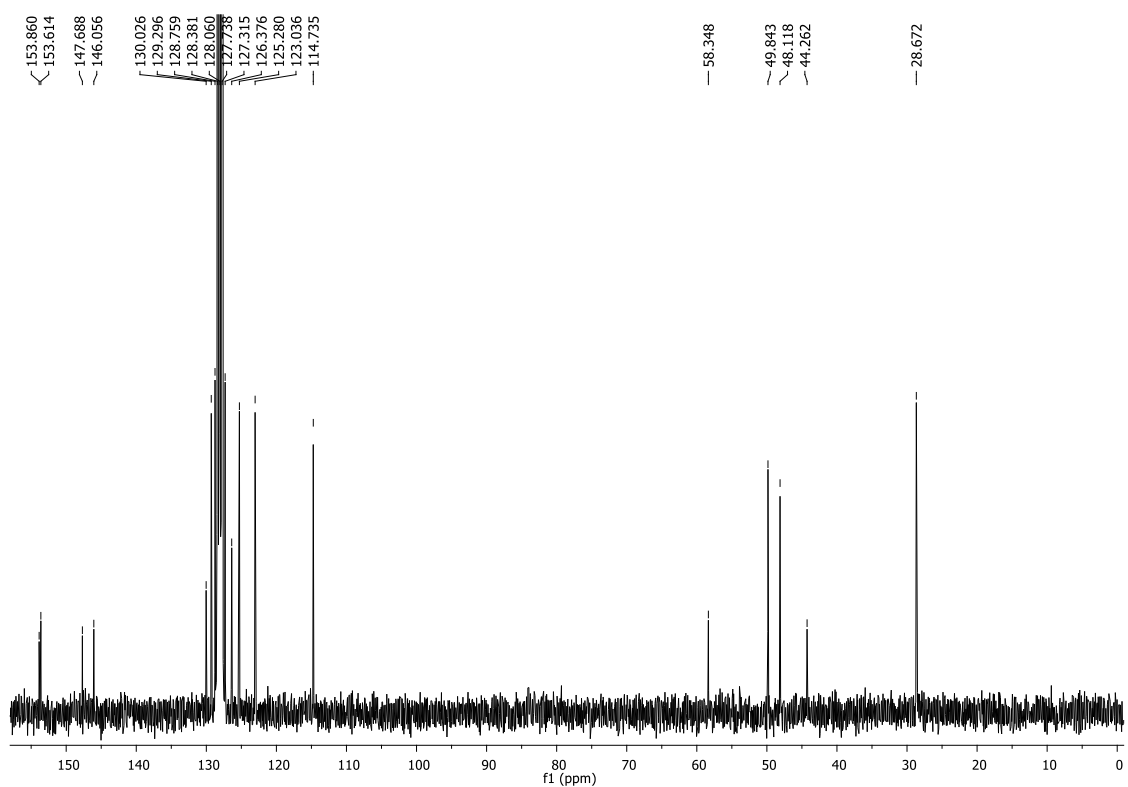




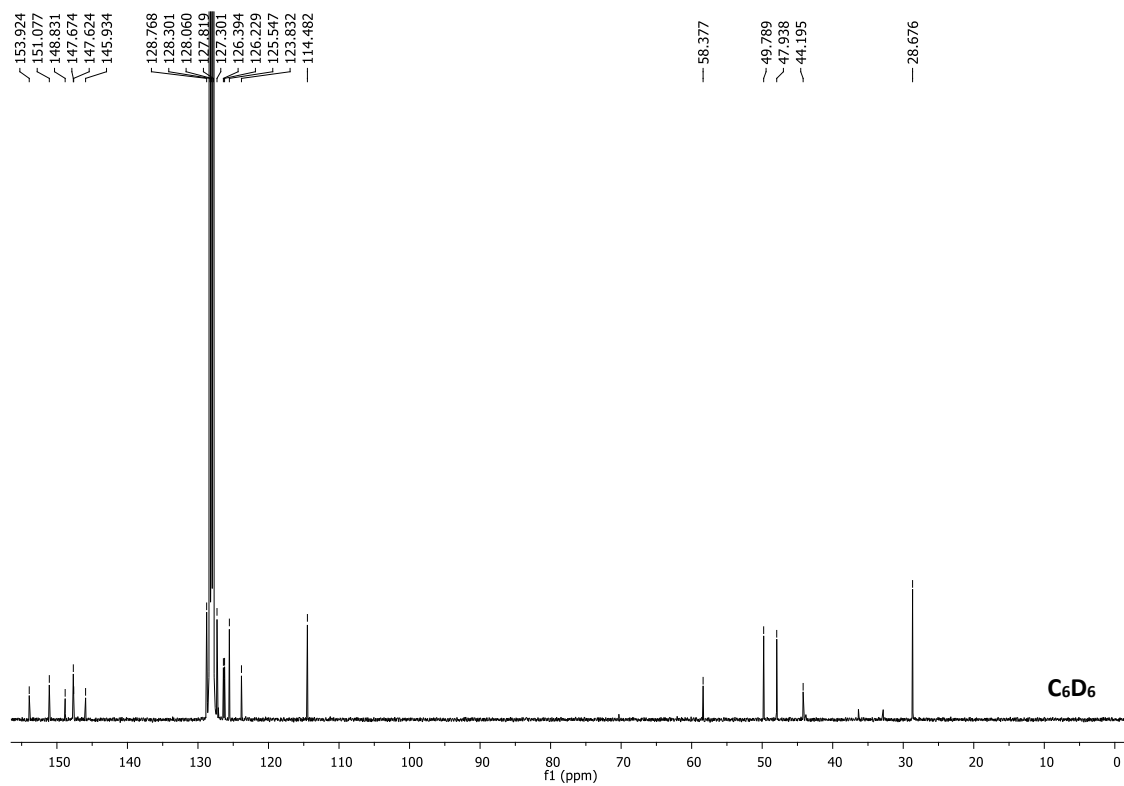
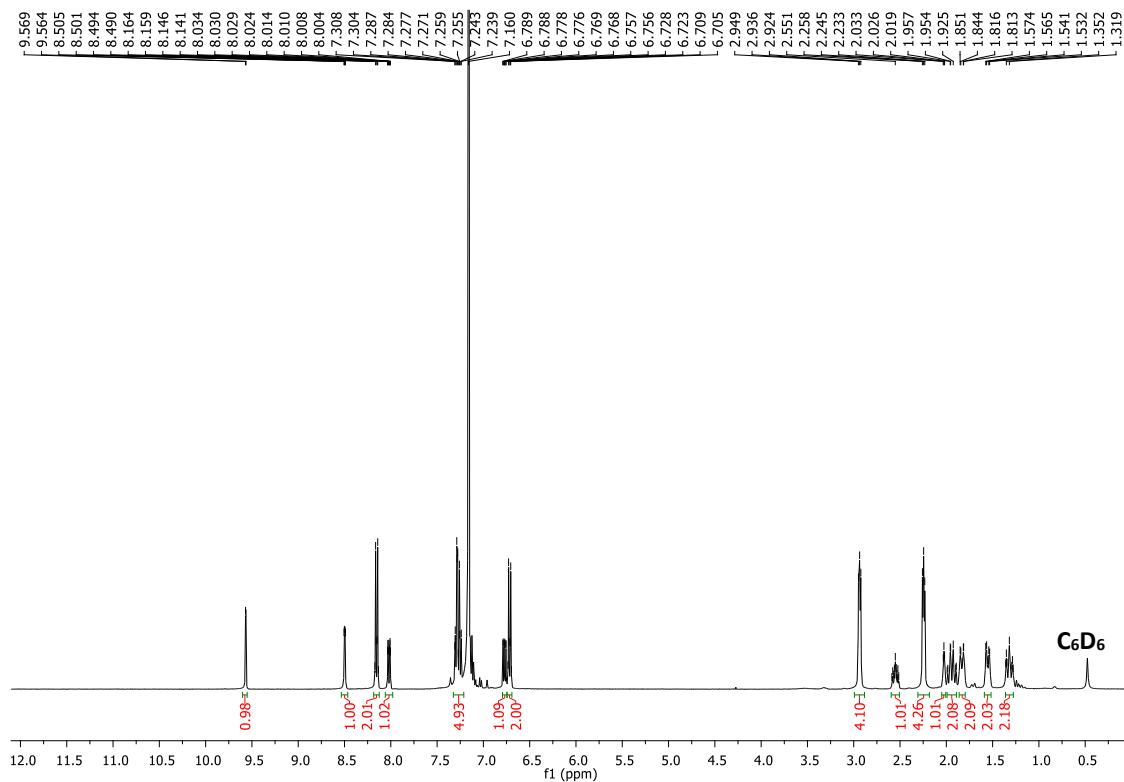


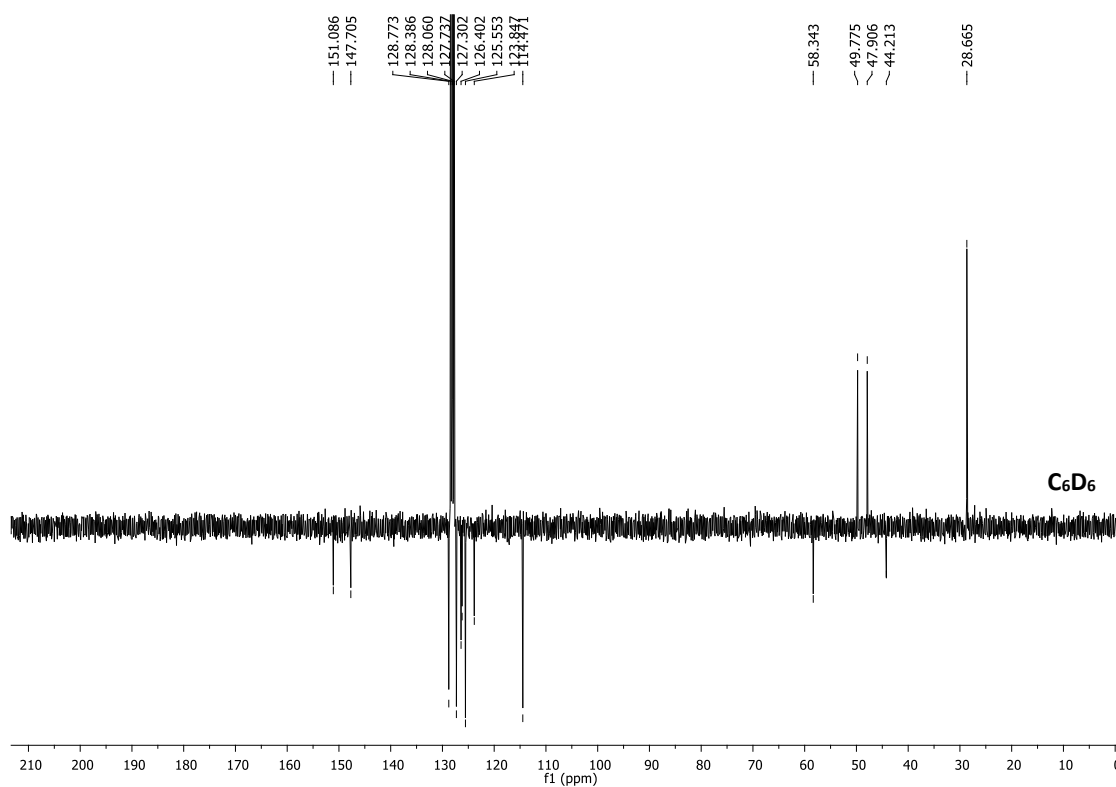
1-((1S,4S)-4-phenylcyclohexyl)-4-((E)-phenyldiazenyl)phenyl)piperazine, hydrochloride salt (9b)



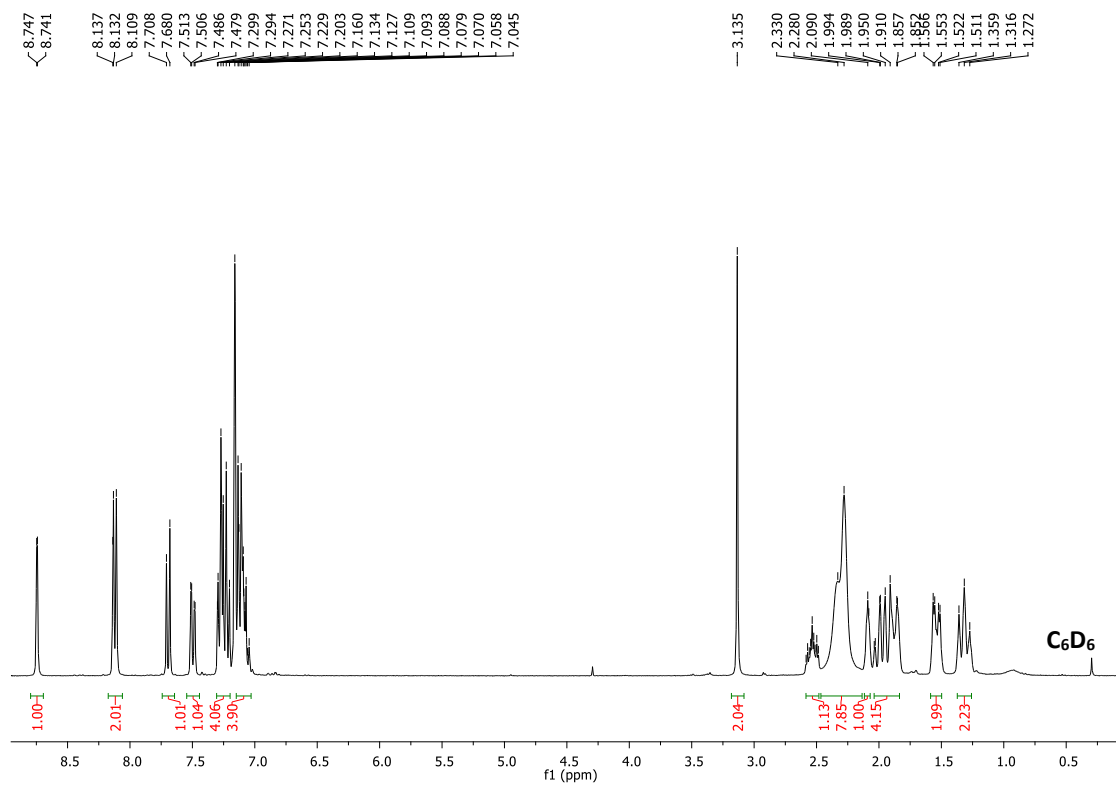


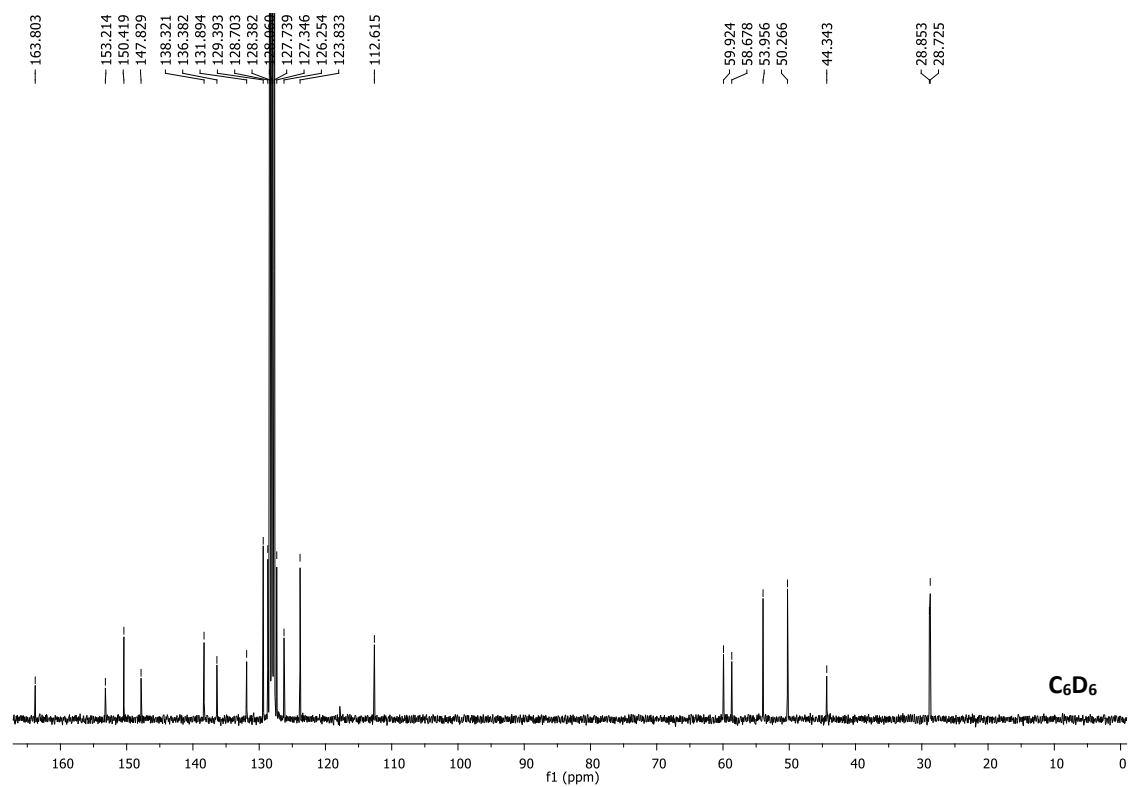
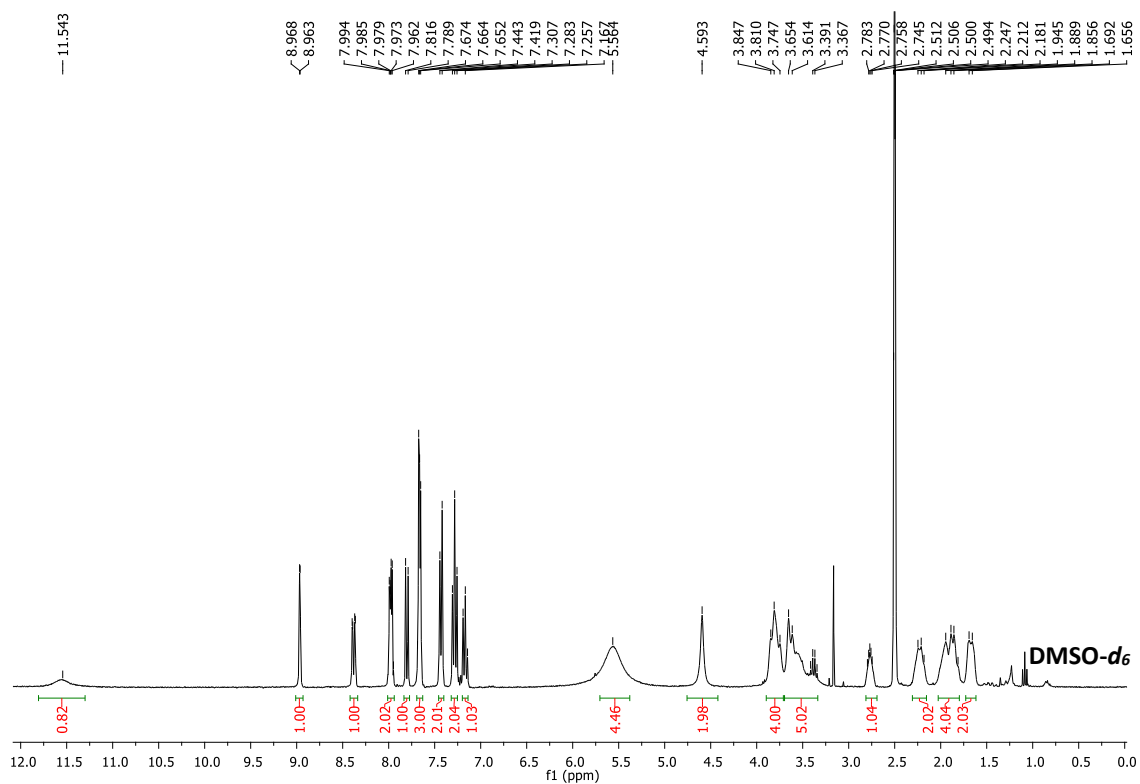
**1-((1*S*,4*S*)-4-phenylcyclohexyl)-4-(4-((*E*)-pyridin-3-yl diazenyl)phenyl)piperazine,
double hydrochloride salt (9c)**

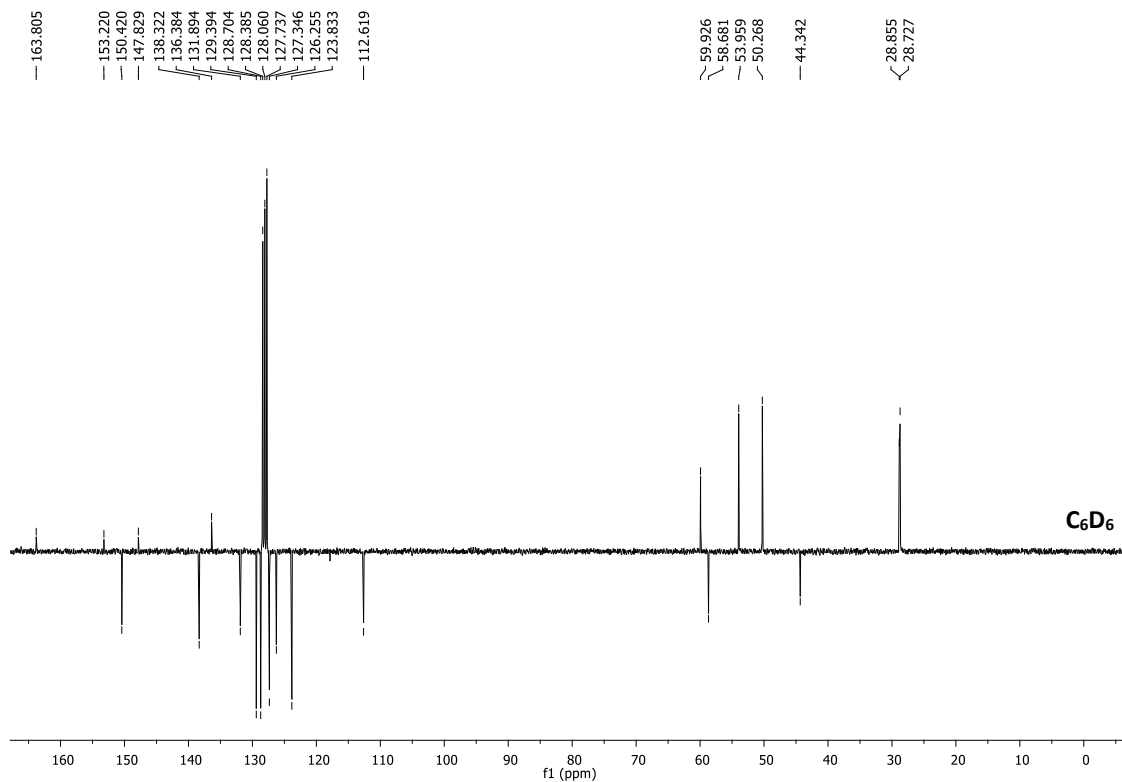




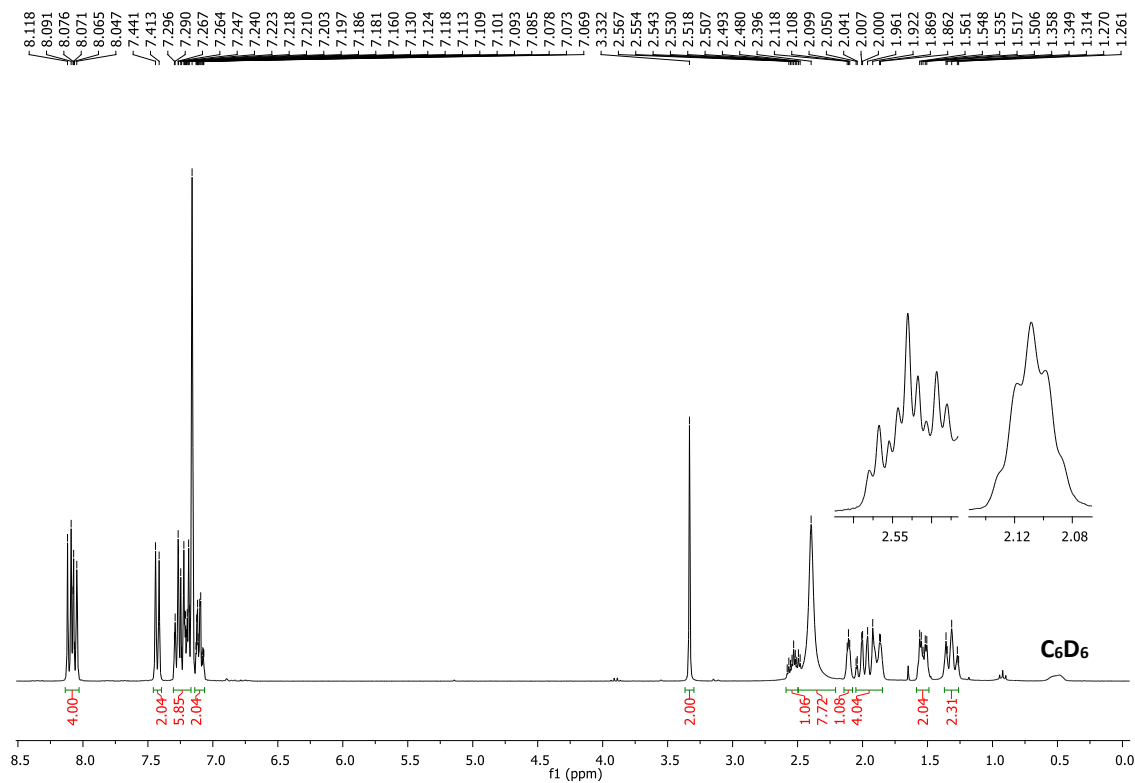
1-((1*S*,4*S*)-4-phenylcyclohexyl)-4-((6-((*E*)-phenyldiazenyl)pyridin-3-yl)methyl)piperazine, double hydrochloride salt (9d)

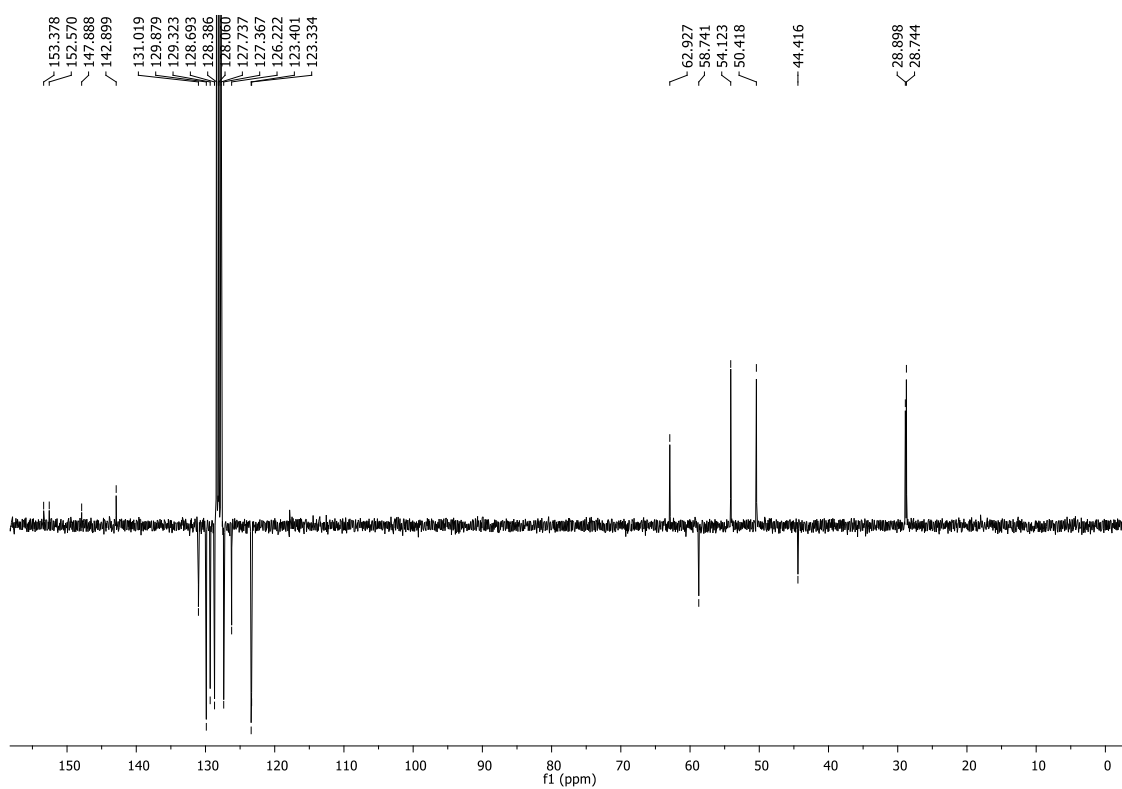
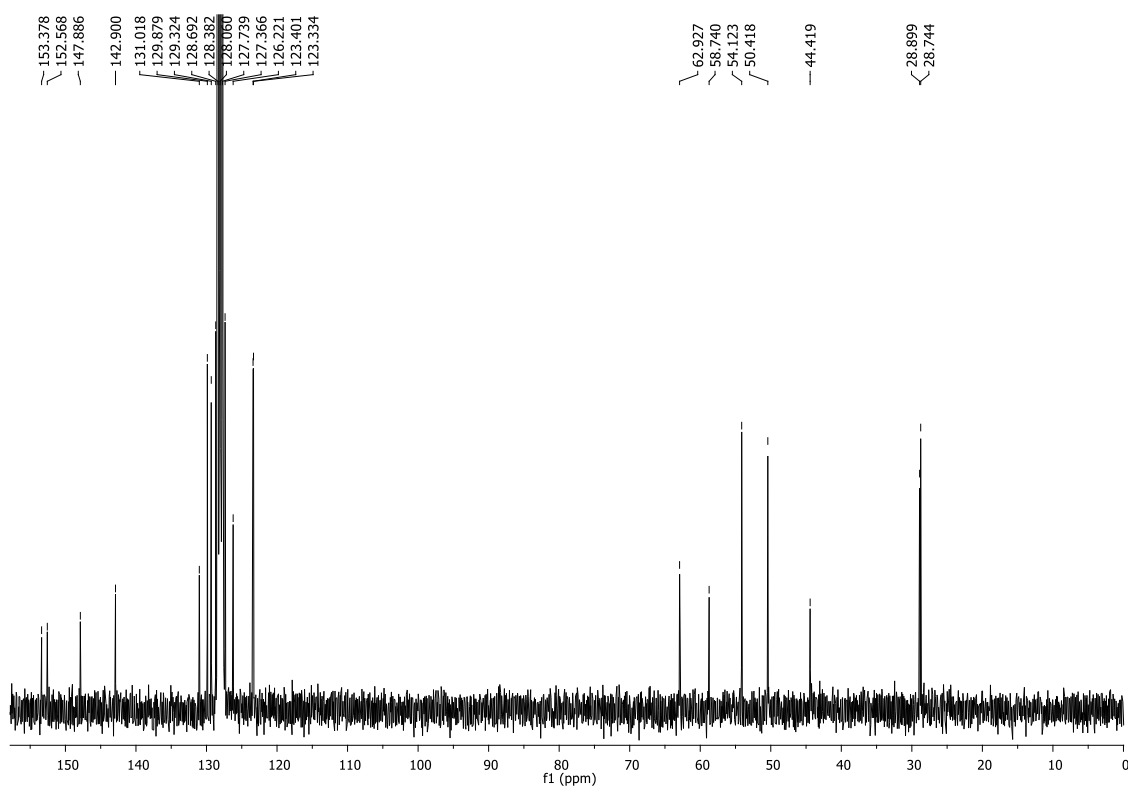






1-((1S,4S)-4-phenylcyclohexyl)-4-(4-((E)-phenyldiazenyl)benzyl)piperazine, double hydrochloride salt (9e)





4. X-Ray crystal structure report

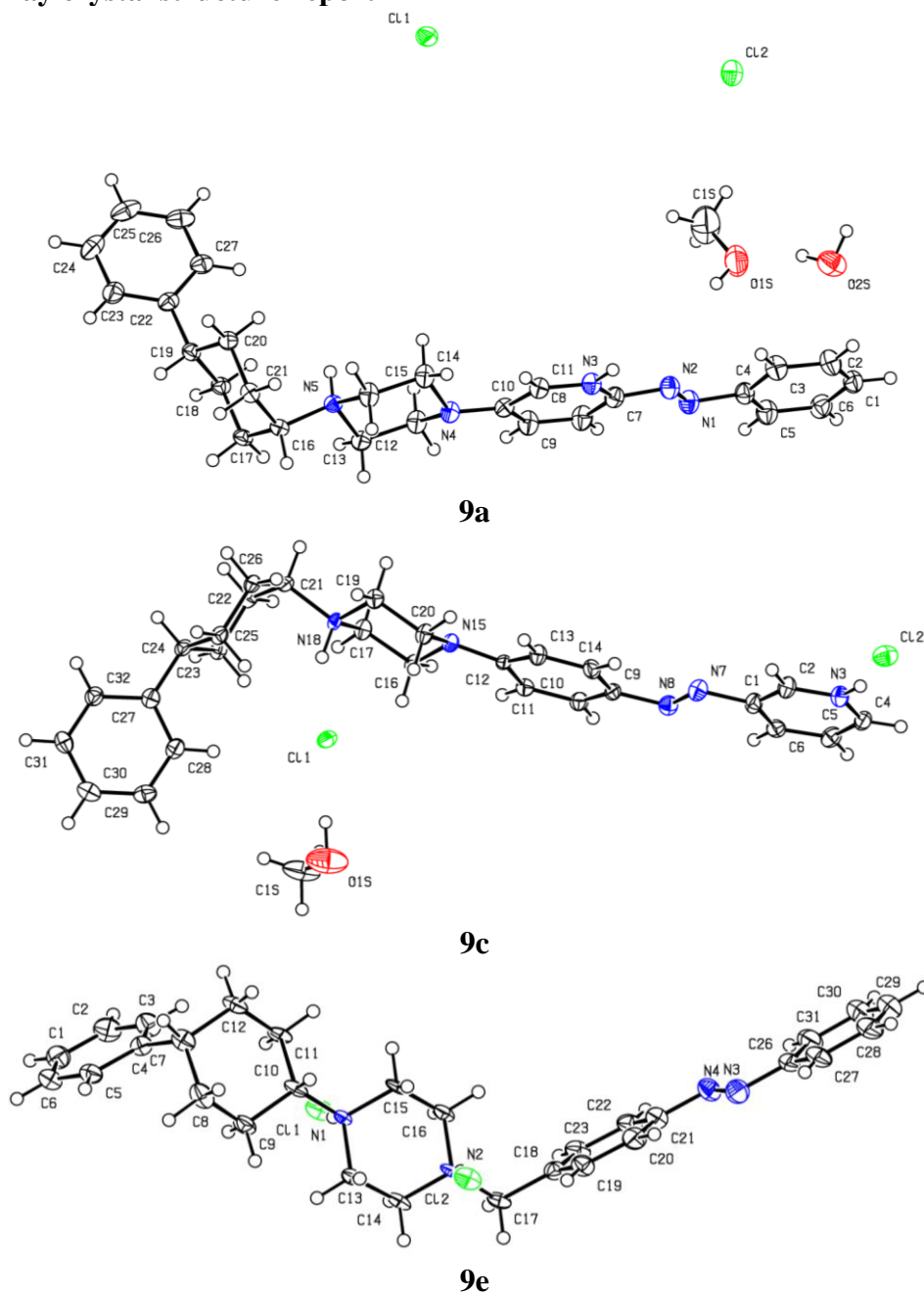


Figure S2. X-ray crystal structures of **9a** (CCDC 1906657), **9c** (CCDC 1906654) and **9e** (CCDC 1906656) shown as ORTEP with ellipsoids drawn at the 50% probability level. Hydrogen atoms (white spheres, arbitrary radius) were located in the difference Fourier map and refined freely. The analysis reveal that for all compounds, the stable *E*-configuration of the diazobenzene were present. The cyclohexyl ring adopts a chair conformation (cis-diastereomer). CCDC codes contains the supplementary crystallographic data for this paper. The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures.

5. Photochemical properties of 9a-e.

The photoisomerization was determined using the procedures described by Lutz *et al* (2018)¹ and Schehr *et al* (2019)² with modifications. Briefly, the UV-vis spectra (Tecan Infinite® M1000 PRO) of 500 μ M DMSO solutions of each individual compound maintained in the dark was recorded. Then, the samples were irradiated under 365 nm (5 min) and the UV-vis spectra were recorded immediately. Finally, the previous procedure was performed again with an additional irradiation at 470 nm (5 min) and the UV-vis spectra was recorded immediately (Figure S3). In parallel, the stock solutions of selected compounds were diluted to 0.9 mM and were injected in the UPLC-MS before and after irradiation at 365 nm for 5 min in order to calculate the ratio between *E*- and *Z*-isomers (assigned as $\alpha_{(E)/(Z)}$).

In order to analyze the thermal isomerization ($T_{1/2}$) of compounds **9a**, **9d** and **9e**, their 500 μ M solutions (DMSO) were stored in the dark for at least 48 h. After recording the spectra, the samples were 365 nm irradiated (5 min) and UV-vis spectra were recorded in defined time intervals. The $\lambda_{\max(E)}$ was plotted in function of time and the equation of one-phase association was calculated (GraphPad® Software, v. 5.0, San Diego, CA, US).

For the photostability of **9e**, UV-vis spectra of 500 μ M DMSO was recorded immediately after alternate cycles of irradiation, at 365 nm and 470 nm (5 min each), respectively. Then, the absorbance at 327 nm was plotted as function of the cycle of irradiation.

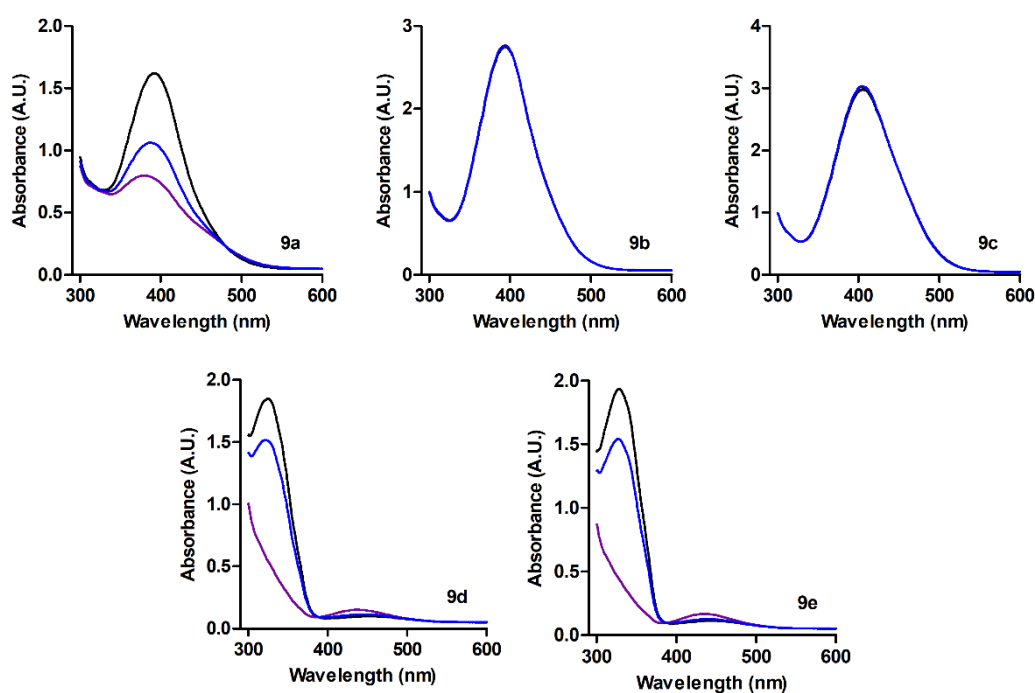


Figure S3. Individual UV-Vis spectra of compounds **9a-e** (DMSO, 500 μ M), in the dark-adapted (black), UV-adapted (magenta, 365 nm), blue-adapted (blue, 470 nm) states.

5.1 UPLC chromatogram

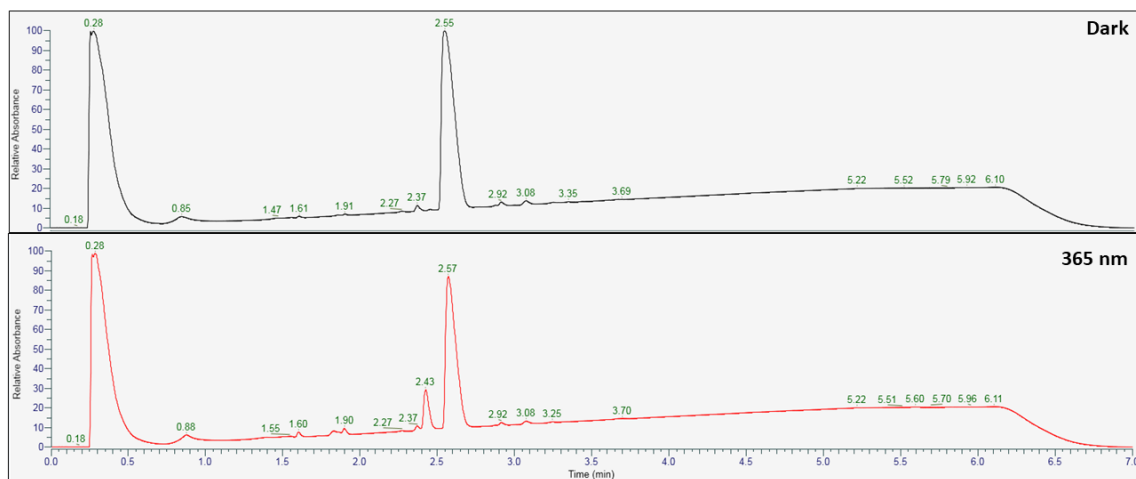


Figure S4. Photoisomerization of **9a**. UPLC chromatogram of the dark-adapted state (black line, 2.55 min [100%]) and 365 nm irradiated (red line, 2.43 min [12%] and 2.57 min [88%]).

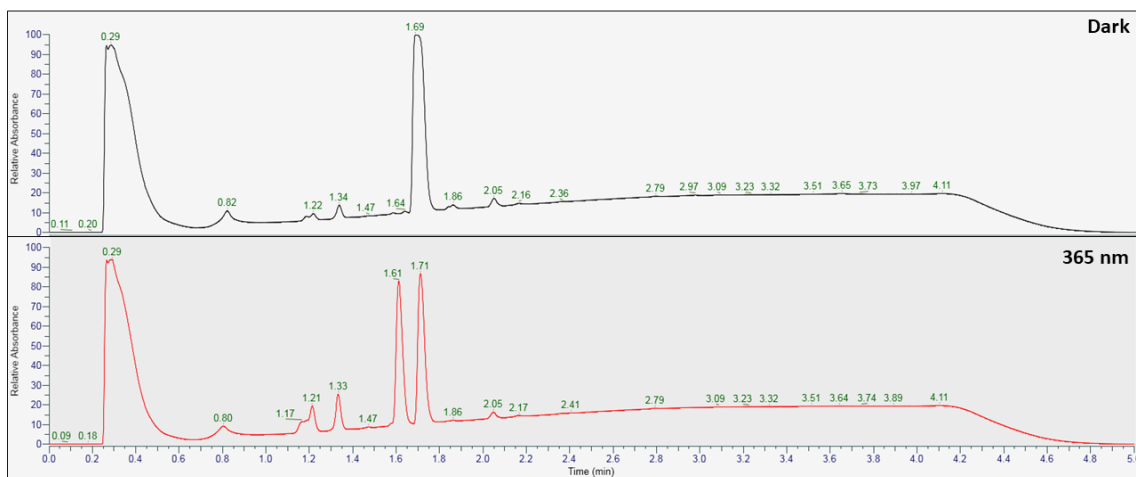


Figure S5. Photoisomerization of **9d**. UPLC chromatogram of the dark-adapted state (black line, 1.69 min [100%]) and 365 nm irradiated (red line, 1.61 min [50%] and 1.71 min [50%]).

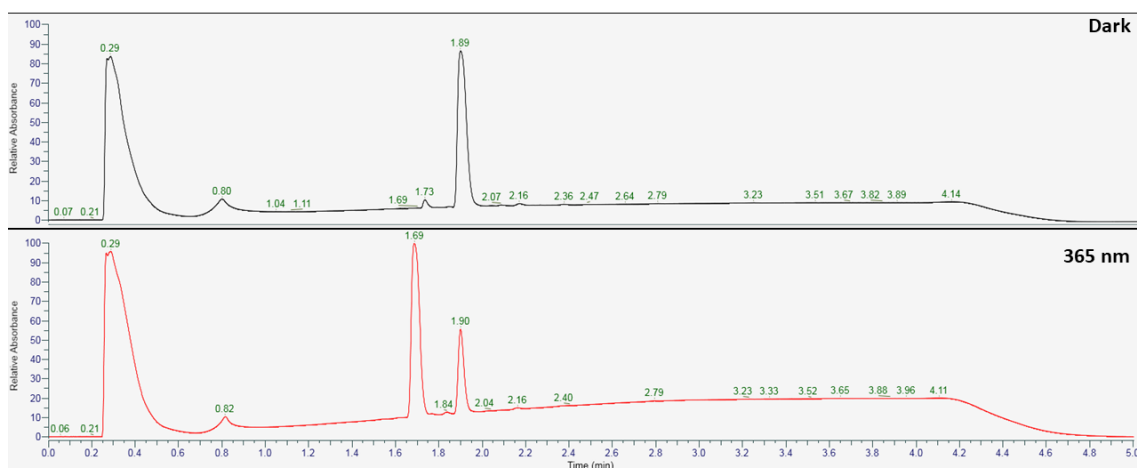


Figure S6. Photoisomerization of **9e**. UPLC chromatogram of the dark-adapted state (black line, 1.69 [3%] and 1.89 min [97%]) and 365 nm irradiated (red line, 1.69 min [75%] and 1.90 min [25%]).

6. Analytical RP-UPLC purity for compounds 9a-e

Table S1. Analytical RP-UHPLC purity of the final compounds

Compound	UHPLC purity (%) ^a
9a	>96
9b	>98
9c	>98
9d	>93
9e	>95

^aDionex ULTIMATE 3000 RSLC chromatography system; Column, Dionex Acclaim[®] RSLC 120 C18, 3.0 x 50 mm, particle size 2.2 μ m, 120 Å pore size; UV Detection, ULTIMATE 3000 RS Photo diode array detector. Eluents, A: water with 0.05% TFA and D: Acetonitrile/water (9/1) with 0.05% TFA. The corresponding UHPLC gradients are given in the characterization of every compound.

7. Biological methodology

7.1 Cell culture and FLIPR assay

Calcium-5 was bought from Molecular Devices LLC. All other chemicals were purchased from Sigma-Aldrich. TRPV6 activity was measured using HEK293 cell line stably overexpressing human TRPV6 as previously reported.³⁻⁵ Stable cells were trypsinized and plated at 75,000 cells/well density onto poly-D-lysine coated 96-well black plates with clear bottom using 100 μ l phenol-red free DMEM supplemented with

10% FBS and 2 mM glutamine without antibiotics. 16 h later the medium was replaced with 90 μL of nominally calcium-free (NCF) loading buffer (modified Krebs buffer containing 117 mM NaCl, 4.8 mM KCl, 1 mM MgCl_2 , 5mM D-glucose, 10 mM HEPES, and calcium-5 fluorescence dye (0.5 vials per 10 ml loading buffer)). Cells were incubated in the NCF-loading buffer at 37 $^{\circ}\text{C}$ for 1 h. Fluorescence Cd^{2+} measurements were carried out using FLIPR_{TETRA} high throughput, fluorescence microplate reader. Cells were excited using a 470–495 nm LED module, and the emitted fluorescence signal was filtered with a 515–575 nm emission filter (manufacturer's guidelines). Stable calcium-free baselines were established for 60 seconds before 10 μL of a 10X compound was added to the cells. Cells were incubated at 37 $^{\circ}\text{C}$ and fluorescence was monitored in the presence of compound for an additional 5 minutes before administration of 100 μL of a 2X substrate (final concentration of 50 μM of CdCl_2). The activity of TPRV6 was measured by calculating the area under the curve of the Cd^{2+} entry traces i.e. following administration of the substrate. Experiments were done with 6 repeats per group. Fluorescence signals were analyzed using the ScreenWorks 3.1.1.8 software (Molecular Devices). Relative IC_{50} values were determined using GraphPad[®] Prism (GraphPad[®] Software, v. 5.0, San Diego, CA, US). Inhibition curves were obtained by non-linear regression using the built-in log(inhibitor) vs. normalized response function.

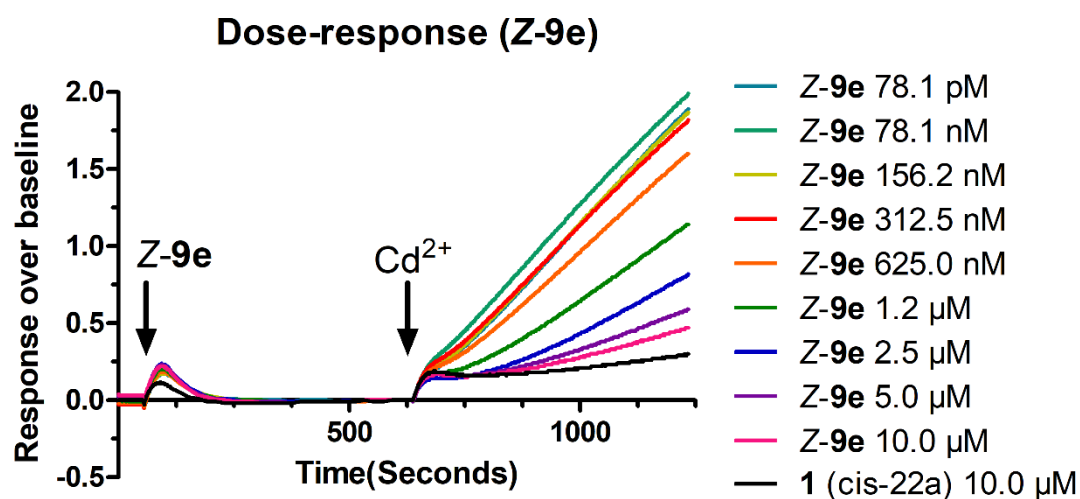


Figure S7. Dose-response curve (Cd^{2+} traces) of Z-9e. Each line represents a concentration of Z-9e ($n = 6/\text{concentration}$). Cis-22a = positive internal control.

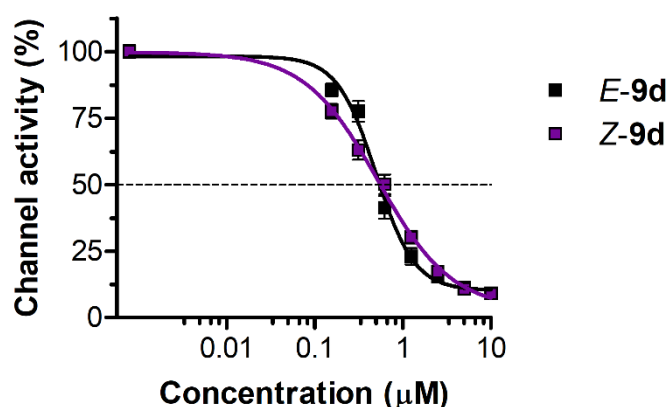


Figure S8. Inhibition curve of Cd^{2+} influx into HEK293-*h*TRPV6 cells by *E/Z*-**9d** (black and magenta squares, respectively). Data shown are mean \pm SEM ($n = 6/\text{concentration}$) from 3 independent experiments.

7.2 Electrophysiology.

HEK293 cells (Leibnitz Institute DSMZ) were cultivated in DMEM medium containing 10% FCS, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. 24 h prior the experiments, the cells were transiently transfected with the peYFP-C1 TRPV6 plasmid by using TransFectinTM Lipid Reagent (Bio-Rad). Patch-clamp experiments were conducted in the whole-cell configuration at room temperature (20-24°C) with an Ag/AgCl reference electrode. Voltage ramps from -90 to +90 mV over a time period of 200 ms were applied every 5 s from a holding potential of 50 mV. The light-activation and -deactivation of **9e** by excitation at 365 nm and 470 nm was induced via a Polychrome IV monochromator (150 W Xe Lamp, Photonics). The internal pipette solution contained: 145 mM Cs-methanesulfonate, 8 mM NaCl, 5 mM MgCl_2 , 10 mM HEPES and 20 mM EGTA, pH 7.2. The extra cellular solution (10 mM Ca^{2+}) consisted of: 145 mM NaCl, 5 mM CsCl, 1 mM MgCl_2 , 10 mM HEPES, 10 mM glucose and 10 mM CaCl_2 , pH 7.4. The currents were leak-corrected by subtraction of the remaining leak currents after La^{3+} (10 μM) block. The liquid junction potential was determined as 12 mV, though the voltages were not adjusted. All experiments were conducted at minimum 2 different days. The application of the corresponding amount (as of the inhibitor) of DMSO in 10 mM Ca^{2+} solution was used as a control. All statistical analyses and graphs were executed with OriginPro software (version 9.1, for Windows, OriginLab, Northampton, MA). To evaluate the effect of the inhibitor, the Wilcoxon signed rank test (2-sided) was conducted.

8. References

- (1) Lutz, T.; Wein, T.; Höfner, G.; Pabel, J.; Eder, M.; Dine, J.; Wanner, K. T. Development of New Photoswitchable Azobenzene Based γ -Aminobutyric Acid (GABA) Uptake Inhibitors with Distinctly Enhanced Potency upon Photoactivation. *J. Med. Chem.* **2018**, *61* (14), 6211–6235.
- (2) Schehr, M.; Ianes, C.; Weisner, J.; Heintze, L.; Müller, M. P.; Pichlo, C.; Charl, J.; Brunstein, E.; Ewert, J.; Lehr, M.; Baumann, U.; Rauh, D.; Knippschild, U.; Peifer, C.; Herges, R. 2-Azo-, 2-Diazocine-Thiazols and 2-Azo-Imidazoles as Photoswitchable Kinase Inhibitors: Limitations and Pitfalls of the Photoswitchable Inhibitor Approach. *Photochem. Photobiol. Sci.* **2019**, *18* (6), 1398–1407.
- (3) Simonin, C.; Awale, M.; Brand, M.; Van Deursen, R.; Schwartz, J.; Fine, M.; Kovacs, G.; Häfliger, P.; Gyimesi, G.; Sithampari, A.; Charles, R. P.; Hediger, M. A.; Reymond, J. L. Optimization of TRPV6 Calcium Channel Inhibitors Using a 3D Ligand-Based Virtual Screening Method. *Angew. Chemie - Int. Ed.* **2015**, *54* (49), 14748–14752.
- (4) Hofer, A.; Kovacs, G.; Zappatini, A.; Leuenberger, M.; Hediger, M. A.; Lochner, M. Design, Synthesis and Pharmacological Characterization of Analogs of 2-Aminoethyl Diphenylborinate (2-APB), a Known Store-Operated Calcium Channel Blocker, for Inhibition of TRPV6-Mediated Calcium Transport. *Bioorg. Med. Chem.* **2013**, *21* (11), 3202–3213.
- (5) Kovacs, G.; Montalbetti, N.; Simonin, A.; Danko, T.; Balazs, B.; Zsembery, A.; Hediger, M. A. Inhibition of the Human Epithelial Calcium Channel TRPV6 by 2-Aminoethoxydiphenyl Borate (2-APB). *Cell Calcium* **2012**, *52* (6), 468–480.