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


# *ortho*-Fluoroazobenzene Derivatives as DNA Intercalators for Photocontrol of DNA and Nucleosome Binding by Visible Light

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## Abbreviations

| | |
|---|---|
| *A* | absorbance |
| APS | ammonium persulfate |
| AU | arbitrary unit |
| bp | base pair |
| CD | circular dichroism |
| d | doublet |
| DIPEA | *N*, *N*-diisopropylethylamine |
| DLS | dynamic light scattering |
| DMAP | 4-(dimethylamino)pyridine |
| dNTP | deoxyribonucleotide triphosphate |
| Dp | *N*,*N*-dimethyl-1,3-diaminopropane |
| $dsDNA_{CT}$ | double-stranded *calf thymus* DNA |
| $dsDNA_{hAT}$ | double-stranded DNA hairpin with the consensus sequence *ATTA* |
| $dsDNA_{hGC}$ | double-stranded DNA hairpin with the consensus sequence *GGCCC* |
| *E.coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid |
| EI | emission intensity, electron ionization |
| EMSA | electrophoretic mobility shift assay |
| ESI-MS | electrospray ionization mass spectroscopy |
| EtBr | ethidium bromide |
| FID | fluorescence intercalator displacement |
| HATU | hexafluorophosphate azobenzotriazole tetramethyl uronium |
| HF | high-fidelity |
| HMBC | heteronuclear multiple bond correlation spectroscopy |
| HRMS | high resolution mass spectroscopy |
| HSQC | heteronuclear single quantum correlation spectroscopy |
| $K_D$ | binding affinity constant |
| kDa | kilodalton |
| m | multiplet |
| n.c. | non-calculated |
| NMR | nuclear magnetic resonance |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| PMSF | phenylmethane sulfonyl fluoride |
| ppm | part per million |
| Py | pyrrole |
| $R_f$ | retention factor |
| RP-HPLC | reverse phase-high performance liquid chromatography |

| | |
|---|---|
| s | singlet |
| t | triplet |
| TEA | triethylamine |
| TBE | tris/borate/EDTA buffer |
| TEMED | tetramethylethylenediamine |
| TMS | trimethylsilyl protecting group |
| TO | thiazole orange |
| Tris | tris(hydroxymethyl)aminomethane |
| UV | ultraviolet |
| Vis | visible |
| $\delta$ | chemical shift |
| $\varepsilon$ | extinction coefficient |
| $\Theta$ | ellipticity |
| $\lambda$ | wavelength |

## Materials and Experimental Procedures

All commercially purchased reagents were used from the following companies without further purification: trichloroacetyl chloride, thionyl chloride, DMSO for molecular biology, potassium chloride, APS, Igepal CA630, EGTA, citric acid, CM-Sephadex, potassium acetate, primers and DNA hairpins from Sigma Aldrich (USA); iron(II) sulfate heptahydrate, acetic anhydride, Tris, boronic acid, 19 : 1 acrylamide : bisacrylamide, TEMED, sucrose, adenine, potassium dihydrogen phosphate and DIPEA from Carl Roth (Germany); sodium hydroxide, potassium hydroxide, EDTA, ethidium bromide, dipotassium hydrogen phosphate, ethanol absolute, PEG 6000, isoamyl alcohol and nitric acid from Merck (Germany); potassium permanganate, *N*,*N*-dimethyl-1,3-diaminopropane (Dp), spermidine, spermine, sodium citrate, calcium chloride and Pd/C from Alfa Aesar (USA); DMF peptide grade and HATU from Iris Biotech (Germany); phusion polymerase and 5X HF buffer from New England Biolabs (USA); 4-bromo-2,6-difluoroaniline from Fluorochem (UK); copper (I) cyanide and heparin sodium salt from TCI (Japan), *N*-methyl pyrrole (Py) and glycerol from Acros Organics (Belgium); DMAP and PMSF from Fluka (USA); agarose, dNTPs and *calf thymus* DNA from Invitrogen (USA); sodium chloride from VWR (USA); sodium dihydrogen phosphate from Grössing GmbH (Austria); netropsin from Santa Cruz Biotechnology (USA); MNase (LS004797) from Worthington (USA); phenol, sodium dodecyl sulfate, RNAse (A3832,0050) and glucose monohydrate from PanReac Applichem ITW Reagents (USA); sodium acetate from Riedel de Haen (Germany); glacial acetic acid and EcoRV from Fisher Scientific (USA) and HT Gel from Biorad (USA). Water was purified with a milli-Q Ultra Pure Water Purification System (TKA, Germany).

### *Nuclear Magnetic Resonance Spectroscopy (NMR)*

All NMR spectra were automatically measured at 300 K either in a Bruker AV III HD 300 MHz at a frequency of 300 MHz ($^{1}$H) or 75 MHz ($^{13}$C) or in a Bruker AV III HD 500 MHz at a frequency of 500 MHz ($^{1}$H) or 125 MHz ($^{13}$C). All chemical shifts ($\delta$) are relative to tetramethylsilane (TMS), i.e. $\delta$(TMS) = 0 ppm. As internal standards, deuterated chloroform ($CDCl_3$), dimethyl sulfoxide (DMSO) with TMS were used. Solvent shifts (ppm): $\delta(CHCl_3)$ = 7.26 ppm ($^{1}$H), $\delta(CHCl_3)$ = 77.16 ppm ($^{13}$C), $\delta$(DMSO) = 2.50 ppm ($^{1}$H), δ(DMSO) = 39.52 ppm ($^{13}$C).[i] Coupling constants are given in Hz. The assignment of each signal was based on two-dimensional nuclear magnetic resonance spectroscopy (2D NMR), i.e. heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation spectroscopy (HMBC). Unless otherwise noted, the NMR tubes were Wilmad NMR tubes (WG-5-7E; OD: 4.947±0.019 mm; ID: 4.1 mm; length 7). For analytical purposes the photoswitchable *ortho*-fluorazobenzene-pyrrole hybrids were fully characterized as their *E*-isomers. The symbol * represents *Z*-isomer signals in the spectra. For the isomerization studies also the *Z*-isomer was analyzed.

### *High Performance Liquid Chromatography (HPLC)*

For preparative purposes, a PLC 2020 Personal Purification System (Gilson) with a preparative Nucleodur C18 HTec-column (5 µm, 250 x 16 mm; Macherey & Nagel) using an isocratic regime during the first five minutes, for column equilibration, followed by a linear gradient 5% to 60% B in 40 min for compounds **2** and **17** and a linear gradient 5% to 75% B in 30 min for compound **3** with a flow rate of 10 mL/min at 25°C was used. The detection was carried out by measuring the absorption at wavelengths: 220 nm and 260 nm. Milli-Q water (A) and MeCN (B) were employed as eluents with an addition of 0.1% of TFA in both solvents.

For analytical purposes, an Agilent 1260 Infinity II HPLC-System (Agilent Technologies) with either an EC 125/2 Nucleodur 100-C18 ec column (Macherey & Nagel) with a flow rate of 0.2 mL/min for purity analysis or an eclipse XDB-C18 (5 µm, 4.6 x 150 mm, Agilent) with a flow rate of 1.0 mL/min for switching studies using with an isocratic regime during the first five minutes, for column equilibration, followed by a linear gradient 5% to 75% B in 30 min at 55°C was used.

## *Mass Spectrometry*

Electrospray ionization mass spectrometry (ESI-MS) was performed on a Finnigan LTQ-FT (Thermo Fischer Scientific). EI-MS was performed on an AccuTOF GCv (JEOL).

## *UV-Vis Spectroscopy*

UV-Vis measurements were performed on a Tecan 20M at room temperature using a quartz cuvette (Hellma Analytics; 104-QS) with a pathlength of 1 cm in a volume of 1 mL. The represented measurements were always buffer substracted.

## *Fluorescence Spectroscopy*

Fluorescence measurements were performed on a Jasco FP-6500 spectrofluorometer equipped with a Thermo Haake WKL 26 water recirculator at 20°C. Unless otherwise noted, fluorescence cuvette (Hellma Analytics; 104F-QS) was used. The measurements were performed with the following settings: increment: 1.0 nm; integration time: 0.2 s; speed: 500 nm/min; sensitivity: medium; excitation slit width: 3 nm; emission slit width: 5 nm; excitation wavelength 490 nm. The emission spectra were recorded from 510 to 700 nm at 20°C. From all spectra, the corresponding background signal was subtracted. Measurements were performed in triplicates. The represented measurements were always buffer substracted.

## *Circular Dichroism*

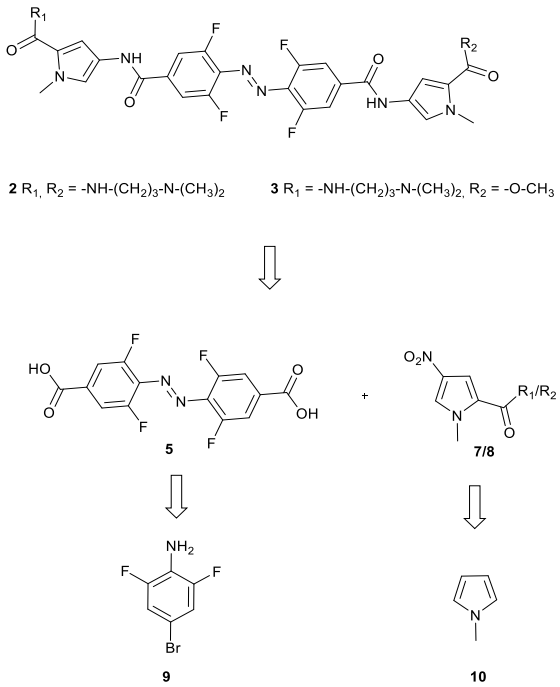
CD titration measurements were performed on a Jasco J-810 Spectropolarimeter equipped with a Jasco CDF-426S peliter controller and a Thermo Haake WKL 26 water recirculator at 20°C using a CD cuvette (Hellma Analytics; QS 0.200). The settings were: range from 550-230 nm, speed of 100 nm/min, response of 0.25 s, band width of 2 nm and a data pitch of 1 nm. The represented measurements were always buffer substracted.

## *Dynamic Light Scattering*

Dynamic Light Scattering (DLS) measurements were performed on a Malvern Instruments (UK) Zetasizer Nano ZS equipped with a 10 mW HeNe laser at a wavelength of 633 nm. Measurements were performed at 25°C using an UVette (Eppendorf; 0030106300). The settings were: material: protein (RI 1.450, absorbance 0.001); dispersant: viscosity 0.8812 cP, RI 1.331; equilibration time: 180 s; cell: disposable cuvette ZEN0040; measurement: measurement angle: 173° backscatter (NIBS default), measurement duration: number of runs 10, run duration 10 s. The corresponding hydrodynamic diameter were then analyzed with respect to their number percentage by DLS.

## Synthesis

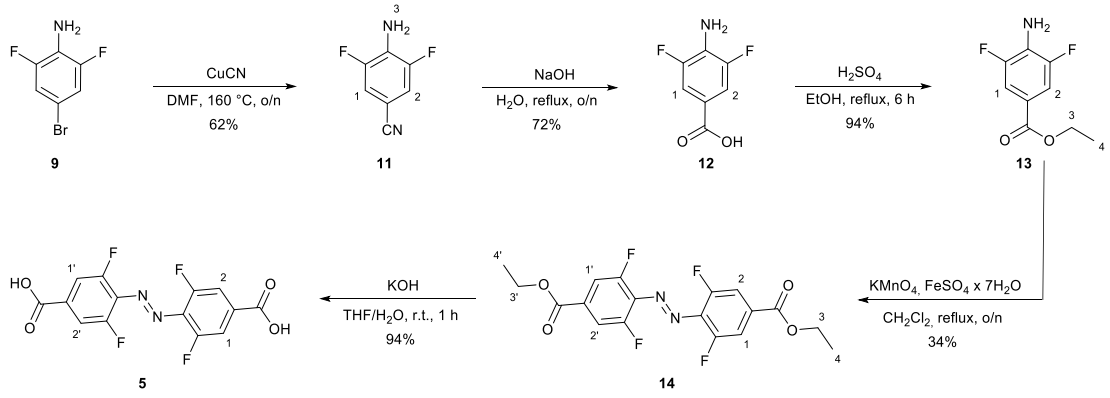
The retrosynthesis of the photoswitches is illustrated for the *E*-isomer because it is the most stable thermodynamically.




**Scheme S1:** Retrosynthetic approach for the preparation of the flourazobenzene derivatives $F_4$Azo-(PyDp)$_2$ (**2**) and $F_4$Azo-(PyDp)(PyOMe) (**3**).

## *Synthesis of the Symmetrical ortho-fluorazobeneze-4,4'-dicarboxylic acid ($F_4$Azo-diacid, **5**)*

This synthesis was reported before by Hecht *et al.*[ii,iii] Such procedure is based in the preparation of the symmetrical *ortho*-fluorazobeneze-4,4'-diester ($F_4$-diester, **14**).[ii] We followed the same approach with minor modifications.




**Scheme S2**: Synthesis route of $F_4$Azo-diacid (**5**) starting from 4-bromo-2,6-difluoroaniline (**9**).

***4-amino-3,5-difluorobenzonitrile (11)*:** 4-bromo-2,6-difluoroaniline (**9**, 5.00 g, 24.0 mmol) and CuCN (6.45 g, 72.0 mmol) were dissolved in DMF (50 mL) and heated to 160°C for 16 h. The mixture was poured into saturated $Na_2CO_3$ (250 mL), filtered and the precipitate washed with EtOAc. The organic phases were washed with $NH_3$ 12% aqueous solution and dried over $MgSO_4$. The crude product was purified by column chromatography ($CH_2Cl_2$/*n*-pentane 2:1) to yield the desired product (**11**, 2.29 g, 14.9 mmol, 62 %) as a white solid. **$R_f$** = 0.39 ($CH_2Cl_2$/*n*-pentane 2:1); **δH(300 MHz; $CDCl_3$; $Me_4Si$)** 7.13 (2 H, dd, $^3J_{HF}$ 6.0, $^4J_{HH}$ 2.3, H-1 and H-2), 4.29 (2 H, br s, $NH_2$); **δC (75 MHz; $CDCl_3$; $Me_4Si$)** 152.3 ($C_{arom.}$), 149.0 ($C_{arom.}$), 129.8 ($C_{arom.}$), 118.0 (CN), 115.7 (CH), 115.4 (CH); 98.4 ($C_{arom.}$) **HRMS-EI$^+$ (*m/z*):** $[M]^+$ calcd for $C_7H_4F_2N_2$, 154.03425; found, 154.03252.

***4-amino-3,5-difluorobenzoic acid (12)*:** 4-amino-3,5-difluorobenzonitrile (**11**, 2.25 g, 14.6 mmol) was suspended in 1M NaOH (80 mL) and refluxed overnight. The reaction mixture was cooled to r.t. and acidified with 1M HCl. The resulting precipitate was dissolved in EtOAc, dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to yield the desired product (**12**, 2.20 g, 12.7 mmol, 87 %) as a white solid. **$R_f$** = 0.93 (EtOAc/formic acid 1:0.02); **δH(300 MHz; [D6]DMSO; $Me_4Si$)** 12.68 (1 H, s, COOH), 7.39 (2 H, dd, $^3J_{HF}$ 7.2, $^4J_{HH}$ 2.4, H-1 and H-2), 6.05 (2 H, s, $NH_2$); **δC (75 MHz; [D6]DMSO; $Me_4Si$)** 166.0 (COOH), 151.3 ($C_{arom.}$), 148.2 ($C_{arom.}$), 130.6 ($C_{arom.}$), 115.7 ($C_{arom.}$), 112.3 (CH), 112.1 (CH); **HRMS-ESI$^-$ (*m/z*):** $[M]^-$ calcd for $C_7H_4F_2N_1O_2$, 172.0216; found, 172.0217.

***ethyl 4-amino-3,5-difluorobenzoate (13)*:** 4-amino-3,5-difluorobenzoic acid (**12**, 1.90 g, 11.0 mmol) was dissolved in EtOH (40 mL) and conc. $H_2SO_4$ (4 mL) and refluxed for 5 h. Conc. $H_2SO_4$ was added and the reaction mixture refluxed for 1 h. The mixture was neutralized with sat. $NaHCO_3$, extracted with $CH_2Cl_2$, the organic phases dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to yield the desired product (**13**, 2.07 g, 10.3 mmol, 94 %) as a pale brown solid. **$R_f$** = 0.29 ($CH_2Cl_2$/*n*-pentane 1:1); **δH(300 MHz; $CDCl_3$; $Me_4Si$)** 7.53 (2 H, dd, $^3J_{HF}$ 7.2, $^4J_{HH}$ 2.1, H-1 and H-2), 4.33 (2 H, q, $^3J_{HH}$ 7.1 Hz, H-3), 3.86 (2 H, br s, $NH_2$), 1.37 (3 H, t, $^3J_{HH}$ 7.1, H-4); **δC (75 MHz; [D6]DMSO; $Me_4Si$)** 165.3 (CO), 152.4 ($C_{arom.}$), 149.3 ($C_{arom.}$), 128.9 ($C_{arom.}$), 118.8 ($C_{arom.}$), 112.8 (CH), 112.6 (CH), 61.2 ($CH_2$), 14.5 ($CH_3$); **HRMS-ESI$^+$ (*m/z*):** $[M]^+$ calcd for $C_9H_9F_2N_1O_2H$, 202.0674; found, 202.0675.

***diethyl 4,4'-(diazene-1,2-diyl)-bis(3,5-difluorobenzoate) (14)*:** ethyl 4-amino-3,5-difluorobenzoate (**13**, 1.36 g, 6.76 mmol) was dissolved in $CH_2Cl_2$ (115 mL) and $KMnO_4$ (11.5 g, 73.0 mmol) and $FeSO_4$ x $7H_2O$ (11.5 g, 41.2 mmol) were added. The reaction mixture was refluxed overnight and filtered over celite. The solvent was removed under reduced pressure and the crude purified by column chromatography ($CH_2Cl_2$/*n*-pentane 1:1) to yield the desired product (**14**, 452 mg, 1.13 mmol, 34 %) as an orange solid. **$R_f$** = 0.75 ($CH_2Cl_2$); **HRMS-ESI$^+$ (*m/z*):** $[M]^+$ calcd for $C_{18}H_{14}F_4N_2O_4H$, 399.0962; found, 399.0963.

***E*-isomer:**

**δH(300 MHz; $CDCl_3$; $Me_4Si$)** 7.74 (4 H, d, $^3J_{HF}$ 8.9, H-1, H-1', H-2 and H-2'), 4.43 (4 H, q, $^3J_{HH}$ 7.1, H-3 and H-3'), 1.43 (6 H, t, $^3J_{HH}$ 7.1, H-4 and H-4'); **δC (75 MHz; $CDCl_3$; $Me_4Si$)** 163.8 (2 C, CO), 156.9 (2 C, $C_{arom.}$), 153.4 (2 C, $C_{arom.}$), 134.0 (2 C, $C_{arom.}$), 133.9 (2 C, $C_{arom.}$), 114.2 (2 C, CH), 113.9 (2 C, CH), 62.3 (2 C, $CH_2$), 14.3 (2 C, $CH_3$).

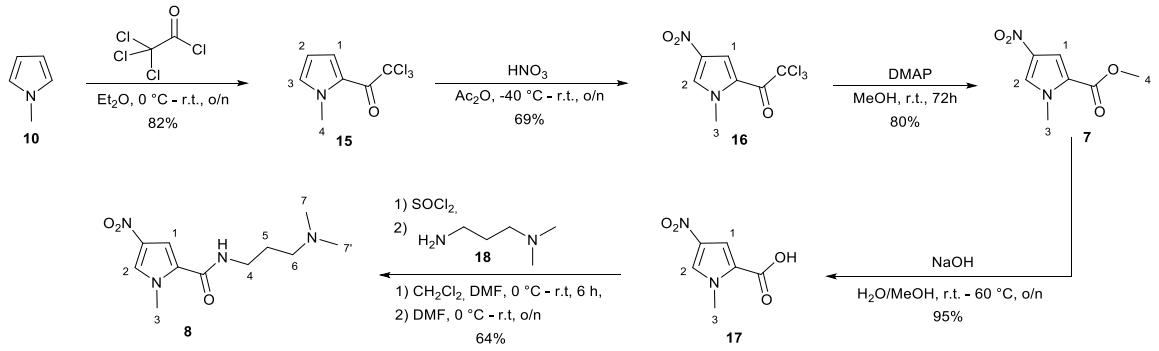
***4,4'-(diazene-1,2-diyl)-bis(3,5-difluorobenzoic acid) (5)*:** diethyl 4,4'-(diazene-1,2-diyl)-bis(3,5-difluorobenzoate) (**14**, 449 mg, 1.13 mmol) was dissolved in THF (25 mL) and KOH (228 mg, 4.06 mmol) dissolved in $H_2O$ (13 mL) was added. The reaction mixture was stirred for 1 h and acidified with 1M HCl. The formed precipitate was filtered and dried on the freeze-dryer overnight to yield the desired product (**5**, 362 mg, 1.06 mmol, 94 %) as a pale orange solid. **$R_f$** = 0.63 (EtOAc/formic acid 1:0.02); **HRMS-ESI$^-$ (*m/z*):** $[M]^-$ calcd for $C_{14}H_5F_4N_2O_4$, 341.0191; found, 341.0203.

***E*-isomer:**

**δH(300 MHz; [D6]DMSO; $Me_4Si$)** 13.84 (1 H, br s, COOH), 7.80 (4 H, d, $^3J_{HF}$ 9.3 Hz, H-1, H-1', H-2 and H-2'); **δC (75 MHz; [D6]DMSO; $Me_4Si$)** 164.5 (2 C, CO), 156.0 (2 C, $C_{arom.}$), 152.5 (2 C, $C_{arom.}$), 135.2 (2 C, $C_{arom.}$), 133.0 (2 C, $C_{arom.}$), 114.0 (2 C, CH), 113.7 (2 C, CH).

## *Synthesis of Pyrrole Monomers Py(Dp) (**8**) and Py(OMe) (**7**)*

This synthesis was reported before by Dervan *et al.*,[iv] Lown *et al.*[v] and Pindur *et al.*,[vi] such procedure is based in the preparation of the nitro pyrrole methylester **7**.[iv] We followed the same approach with minor modifications.




**Scheme S3:** Synthesis route of $O_2N$-Py-OMe (**7**) and $O_2N$-Py-Dp (**8**) starting from *N*-methylpyrrole (**10**).

***2,2,2-trichloro-1-(1-methyl-1H-pyrrol-2-yl)ethan-1-one (15)*:** Under inert atmosphere, to a solution of trichloroacetyl chloride (2.80 mL, 25.0 mmol) in $Et_2O$ (5.00 mL), a solution of *N*-methylpyrrole (2.20 mL, 25.0 mmol) in $Et_2O$ (5.00 mL) was added at 0 °C over 1 h. The reaction mixture was stirred overnight at r.t. and neutralized with $K_2CO_3$ solution. It was extracted with $Et_2O$, dried over $MgSO_4$ and the solvent removed under reduced pressure to yield the desired product (**15**, 4.67 g, 20.6 mmol, 82 %) as brown solid. $\mathbf{R_f}$ = 0.60 (*n*-pentane / EtOAc 1:1); **δH(300 MHz; [D4]MeOD; $Me_4Si$)** 7.46 - 7.42 (2 H, m, H-1 and H-3), 6.30 (1 H, dd, $^3J_{HH}$ 4.3, $^3J_{HH}$ 2.5, H-2), 3.91 (3 H, s, H-4); **δC (75 MHz; [D4]MeOD; $Me_4Si$)** 171.8 (CO), 135.3 (CH), 123.6 (CH), 120.7 ($C_q$), 109.1 (CH), 96.0 ($CCl_3$), 36.2 ($CH_3$); **HRMS-EI$^+$ (*m/z*):** $[M]^+$ calcd for $C_7H_6Cl_3N_1O_1$, 225.95932; found, 225.95978.

***2,2,2-trichloro-1-(1-methyl-4-nitro-1H-pyrrol-2-yl)ethan-1-one (16)*:** Under inert atmosphere, to a solution of 2,2,2-trichloro-1-(1-methyl-1*H*-pyrrol-2-yl)ethan-1-one (1.50 g, 6.62 mmol) in $Ac_2O$ (8.00 mL, 84.6 mmol), conc. $HNO_3$ (0.60 mL, 14.4 mmol) was added at -40°C over 30 min. The reaction mixture was slowly warmed up to r.t. and stirred overnight. It was neutralized with $Na_2CO_3$ solution, extracted with EtOAc, washed with *brine*, the organic phases dried over $MgSO_4$ and the solvent removed under reduced pressure to yield the desired product (**16**, 1.24 g, 4.56 mmol, 69 %) as brown solid. $\mathbf{R_f}$ = 0.16 (*n*-pentane / EtOAc 10:1); **δH(300 MHz; [D6]DMSO; $Me_4Si$)** 8.56 (1 H, d, $^4J_{HH}$ 1.6, H-2), 7.80 (1 H, d, $^4J_{HH}$ 1.6, H-1), 4.00 (3 H, s, H-3); **δC (75 MHz; [D6]DMSO; $Me_4Si$)** 172.8 (CO), 134.2 ($C_q$), 132.5 (CH), 120.6 ($C_q$), 116.3 (CH), 94.5 ($CCl_3$), 50.0 ($CH_3$); **HRMS-EI$^+$ (*m/z*):** $[M]^+$ calcd for $C_7H_6Cl_3N_2O_3$, 270.94440; found, 270.94291.

***methyl 1-methyl-4-nitro-1H-pyrrole-2-carboxylate (7)*:** To a solution of 2,2,2-trichloro-1-(1-methyl-4-nitro-1*H*-pyrrol-2-yl)ethan-1-one (**16** ,4.72 g, 17.4 mmol) in MeOH (58.0 mL) DMAP (212 mg, 1.74 mmol) was added and stirred at r.t. for 72 h. The solvent was removed and the crude product purified by column chromatography (*n*-pentane / EtOAc 4:1 → 1:1) to yield the desired

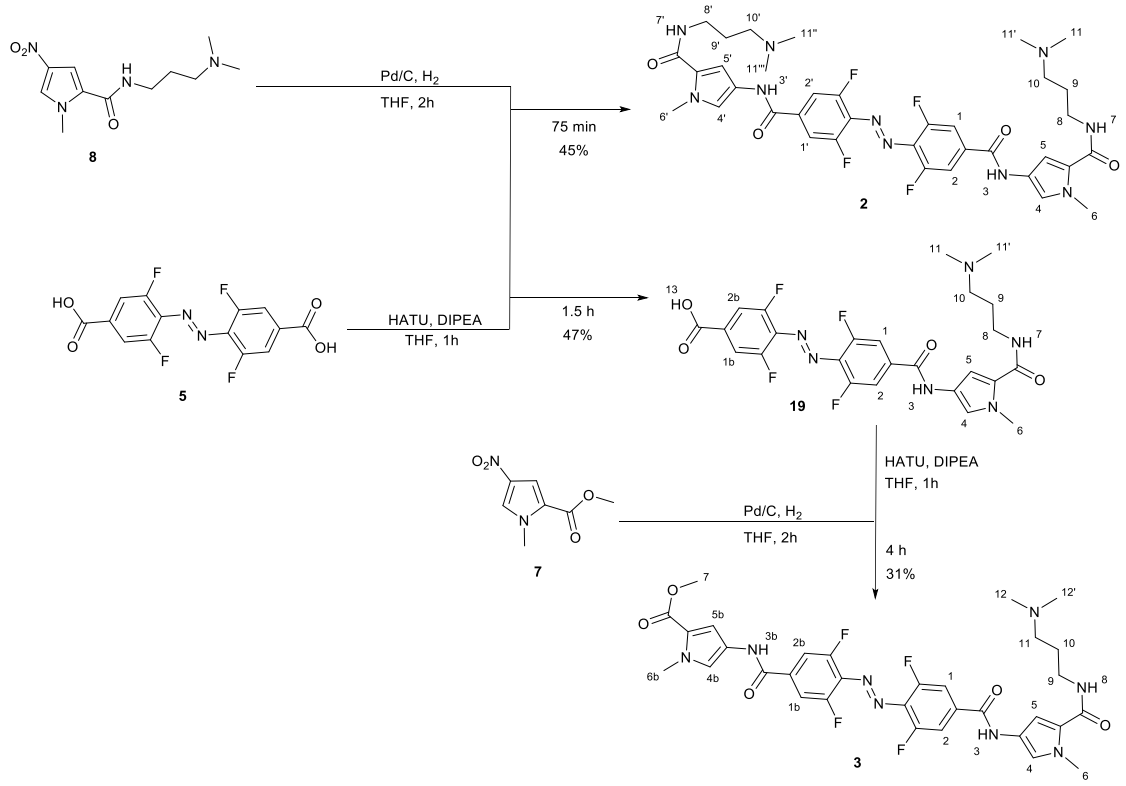
product (**7**, 2.57 g, 14.0 mmol, 80%) as yellow solid. $\mathbf{R_f}$ = 0.16 (*n*-pentane / EtOAc 10:1); **δH(300 MHz; [D6]DMSO; $Me_4Si$)** 8.27 (1 H, d, $^4J_{HH}$ 2.0, H-2), 7.30 (1 H, d, $^4J_{HH}$ 2.0, H-1), 3.92 (3 H, s, H-3), 3.80 (3 H, s, H-4); **δC (75 MHz; [D6]DMSO; $Me_4Si$)** 159.8 (CO), 134.2 ($C_q$), 129.4 (CH), 122.6 ($C_q$), 111.5 (CH), 51.7 ($CH_3$), 37.4 ($CH_3$); **HRMS-ESI$^+$ (*m/z*):** $[M]^+$ calcd for $C_7H_8N_2O_4H$, 185.05623; found, 185.05665.

***1-methyl-4-nitro-1H-pyrrole-2-carboxylic acid (17)*:** To a solution of methyl 1-methyl-4-nitro-1*H*-pyrrole-2-carboxylate (**7** , 2.00 g, 10.9 mmol) in MeOH (25.0 mL) 1M NaOH (15mL) was added and the reaction mixture stirred at r.t. overnight and heated at 60 °C for 1 h. Afterwards, it was extracted with EtOAc and washed with saturated $NaHCO_3$. The aqueous phases were acidified with 2M HCl, extracted with EtOAc, the organic phases dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to yield the desired product (**17**, 1.77 g, 10.4 mmol, 95%) as pale yellow solid. $\mathbf{R_f}$ = 0.33 (EtOAc/formic acid 1:0.02); **δH(300 MHz; [D6]DMSO; $Me_4Si$)** 13.11 (1 H, s, COOH), 8.21 (1 H, d, $^4J_{HH}$ 2.1, H-2), 7.25 (1 H, d, $^4J_{HH}$ 2.1, H-1), 3.91 (3 H, s, H-3); **δC (75 MHz; [D6]DMSO; $Me_4Si$)** 160.9 (CO), 134.0 ($C_q$), 129.1 (CH), 123.8 ($C_q$), 111.4 (CH), 37.4 ($CH_3$); **HRMS-ESI$^-$ (*m/z*):** $[M]^-$ calcd for $C_6H_5N_2O_4$, 169.0255; found, 169.0257.

***N-(3-(dimethylamino)propyl)-1-methyl-4-nitro-1H-pyrrole-2-carboxamide (8)*:** To a solution of 1-methyl-4-nitro-1*H*-pyrrole-2-carboxylic acid (**17**, 674 mg, 3.96 mmol) in $CH_2Cl_2$ (13.5 mL) and DMF (112 µL), $SOCl_2$ (2.88 mL, 39.6 mmol) was added slowly at 0 °C. The reaction mixture was warmed up to r.t. and stirred for another 6 h. The solvent was removed under reduced pressure. At 0 °C under inert atmosphere, the crude was dissolved in DMF (2.70 mL) and *N*,*N*-dimethyl-1,3-diaminopropane (**18**, 1.10 mL, 8.71 mmol) added dropwise. The reaction mixture was warmed to up to r.t. and stirred for 16 h. Deionized water was added to be extracted with EtOAc. The organic phases were washed with saturated $NaHCO_3$, dried over $MgSO_4$, filtered and the solvent removed under reduced pressure. The crude product was purified by column chromatography ($CH_2Cl_2$ / MeOH / TEA 10:1:0.5%) to yield the desired product (**8**, 642 mg, 2.52 mmol, 64%) as pale yellow solid. $\mathbf{R_f}$ = 0.07 ($CH_2Cl_2$ / MeOH / TEA 10:1:0.5%); **δH(300 MHz; [D4]MeOD; $Me_4Si$)** 7.82 (1 H, d, $^4J_{HH}$ 1.9, H-2), 7.27 (1 H, d, $^4J_{HH}$ 1.9, H-1), 3.95 (3 H, s, H-3), 3.33 (2 H, t, $^3J_{HH}$ 7.0, H-4), 2.41 (2 H, t, $^3J_{HH}$ 7.6, H-6), 2.27 (6 H, s, H-7 and H-7'), 1.84 – 1.72 (2 H, m, H-5); **δC (75 MHz; [D4]MeOD; $Me_4Si$)** 162.9 (CO), 136.2 ($C_q$), 128.4 (CH), 127.9 ($C_q$), 108.5 (CH), 58.2 ($CH_2$), 45.4 (2C, $CH_3$), 38.7 ($CH_3$), 37.9 ($CH_2$), 28.1 ($CH_2$); **HRMS-ESI$^+$ (*m/z*):** $[M]^+$ calcd for $C_{11}H_{18}N_4O_3H$, 255.1452; found, 255.1457.

## *Synthesis of photoswitchable ortho-fluorazobenzene-pyrrole hybrids: $F_4$Azo-$(PyDp)_2$ (**2**) and $F_4$Azo-(PyDp)(PyOMe) (**3**)*

This synthesis relies on the condensation reactions between the *$F_4$*Azo-diacid (**5**) and the reduced pyrrole derivatives.




***Scheme S4**:* Synthesis route of photoswitchable fluorazobenzene dimers *$F_4$*Azo-$(PyDp)_2$ (**2**) and *$F_4$*Azo-(PyDp)(PyOMe) (**3**) starting from *$F_4$*Azo-diacid (**5**), $O_2$N-Py-Dp (**8**) and $O_2$N-Py-OMe (**7**).

***4,4'-((4,4'-(ethene-1,2-diyl)bis(3,5-difluorobenzoyl))bis(azanediyl))bis(N-(3-(dimethylamino)propyl)-1-methyl-1H-pyrrole-2-carboxamide) (2)*:** Compound **8**, (78.1 mg, 0.31 mmol) and Pd/C 10% dry (50.0 mg, 64.0% *w/w*) was suspended in THF (3.0 mL) and stirred under $H_2$ atmosphere for 2 h. The reaction mixture was filtered over celite and the solvent removed under reduced pressure. *$F_4$*Azo-diacid (**5**, 50.0 mg, 0.15 mmol) and HATU (117 mg, 0.31 mmol) were dissolved in THF (400 µL) and DIPEA (107 µL, 0.61 mmol) added. The reaction mixture was stirred for 75 min before the reduced pyrrole **8** dissolved in THF (700 µL) was added. After stirring for 4.5 h, the solvent was removed and the crude purified by preparative HPLC (5 - 60% over 40 min) to yield the desired product as its TFA salt (**2**, 68.8 mg, 0.07 mmol, 47%) as orange solid. $t_R$ = 22.6 min (5% to 75% B in 30 min); **HRMS-ESI$^+$ (*m/z*):** $[M]^+$ calcd for $C_{36}H_{42}F_4N_{10}O_4H$, 755.3399; found, 755.3428.

***E*-isomer:**

**δH(500 MHz; [D6]DMSO; $Me_4Si$):** 10.69 (2 H, s, NH-3 and NH-3'), 9.58 (2 H, br s, $NH^+$-12 and $NH^+$-12'), 8.26 (2 H, t, $^3J_{HH}$ 5.9, NH-7 and NH-7'), 7.96 (2 H, s, H-1 and H-1' / H-2 and H-2'), 7.92 (2 H, s, H-1 and H-1' / H-2 and H-2'), 7.33 (2 H, d, $^4J_{HH}$ 1.7, H-4 and H-4'), 6.99 (2 H, d, $^4J_{HH}$ 1.7 Hz, H-5 and H-5'), 3.86 (6 H, s, H-6 and H-6'), 3.26 (4 H, dt, $^3J_{HH}$ 6.3, $^3J_{HH}$ 5.9, H-8 and H-8'), 3.04 – 3.14 (4 H, m, H-10 and H-10'), 2.79 (12 H, s, H-11, H-11', H-11'' and H-11'''), 1.79 – 1.92 (4 H, m, H-9 and H-9'); **δC (125 MHz; [D6]DMSO; $Me_4Si$)** 161.5 (2 C, CO), 160.0 (2 C, CO), 156.2 (2 C, $C_q$), 152.7 (2 C, $C_q$), 138.5 (2 C, $C_q$), 131.9 (2 C, $C_q$), 123.1 (2 C, $C_q$), 121.5 (2 C, $C_q$), 118.7 (2 C, CH), 112.5 (2 C, CH), 112.1 (2 C, CH), 104.5 (2 C, CH), 54.8 (2 C, $CH_2$), 43.4 (4 C, $CH_3$), 36.2 (2 C, $CH_3$), 35.5 (2 C, $CH_2$), 24.7 (2 C, $CH_2$).

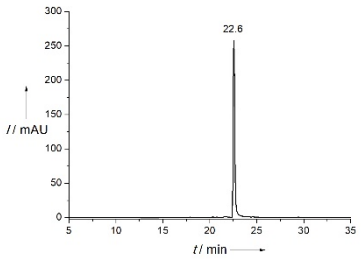



**Figure S1:** HPLC chromatogram of $F_4$Azo-$(PyDp)_2$ (**2**).

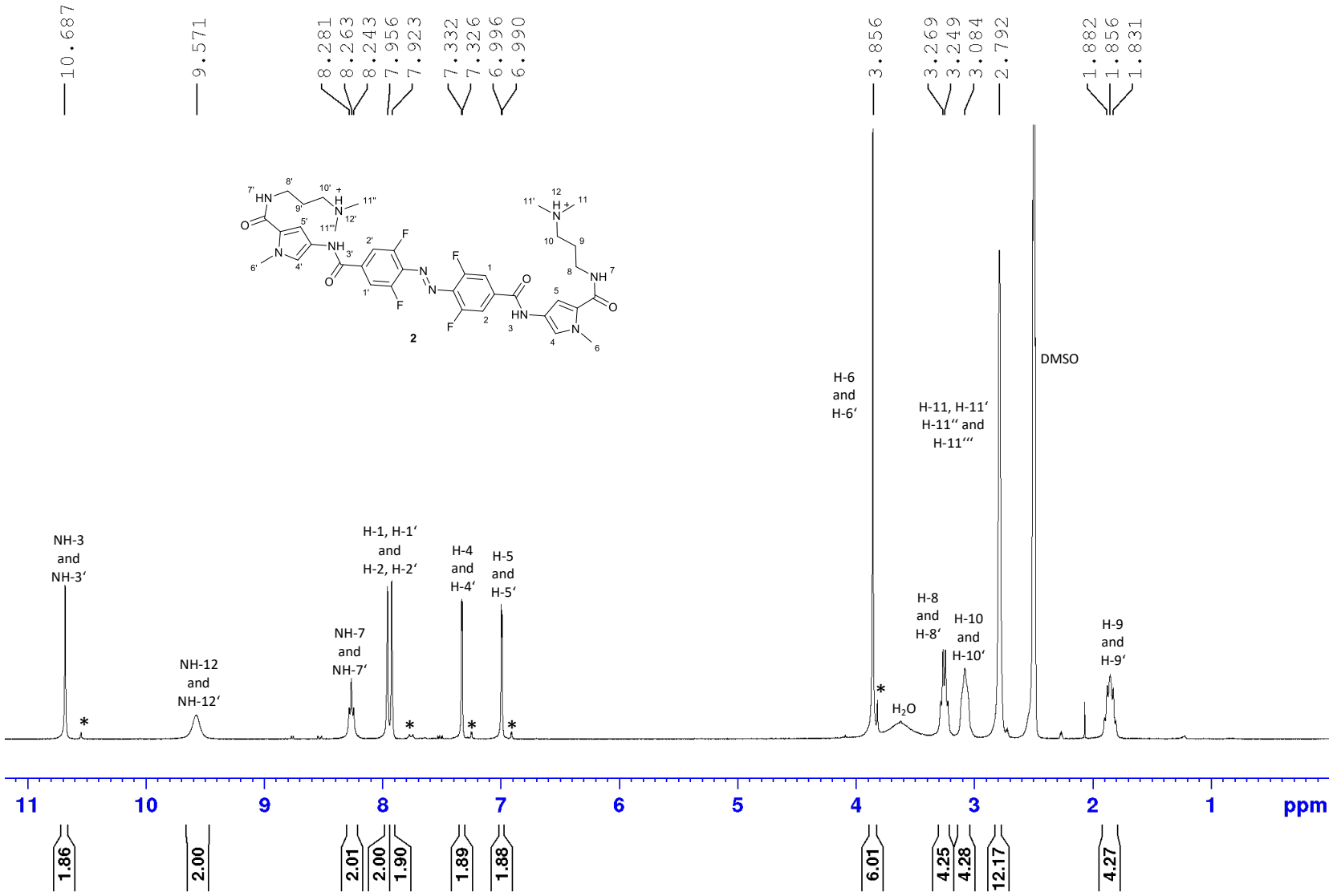



***Figure S2***: *$^1$H-NMR spectrum of trans-$F_4$Azo-$(PyDp)_2$ (**2**).*

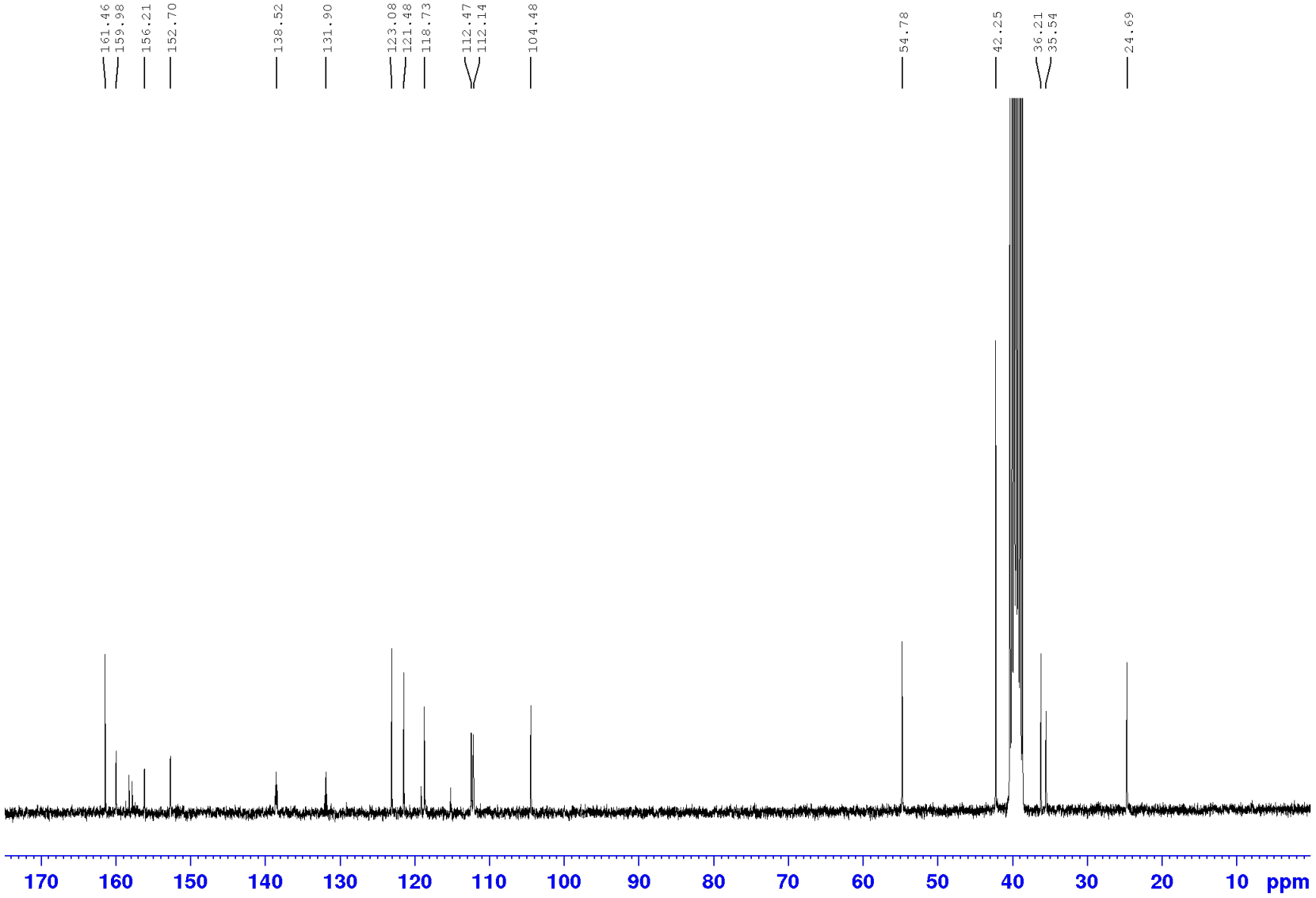



***Figure S3**: $^{13}$C-NMR spectrum of trans-$F_4$Azo-$(PyDp)_2$ (**2**).*

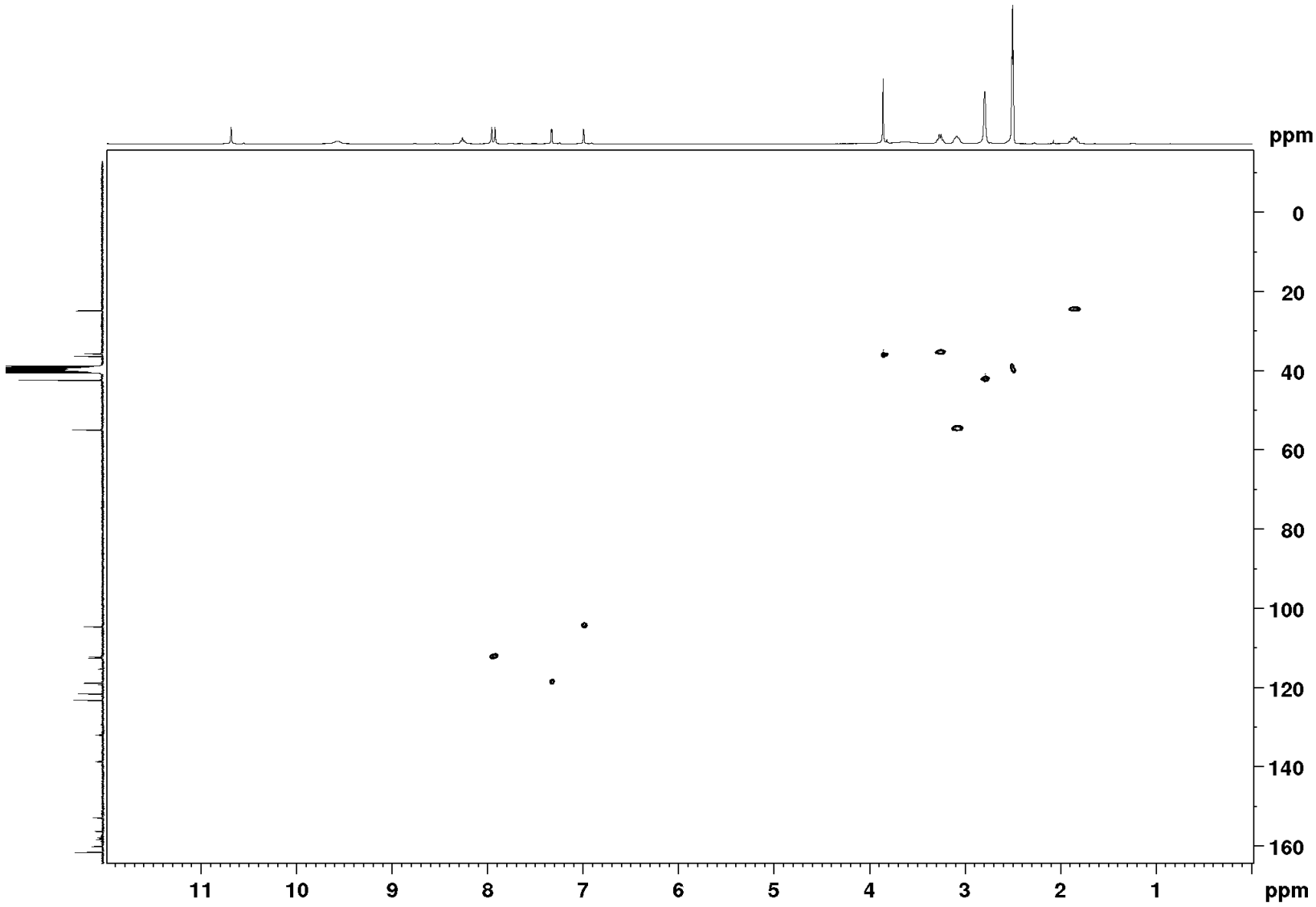



***Figure S4**: HSQC-NMR spectrum of trans-$F_4$Azo-$(PyDp)_2$ (**2**).*

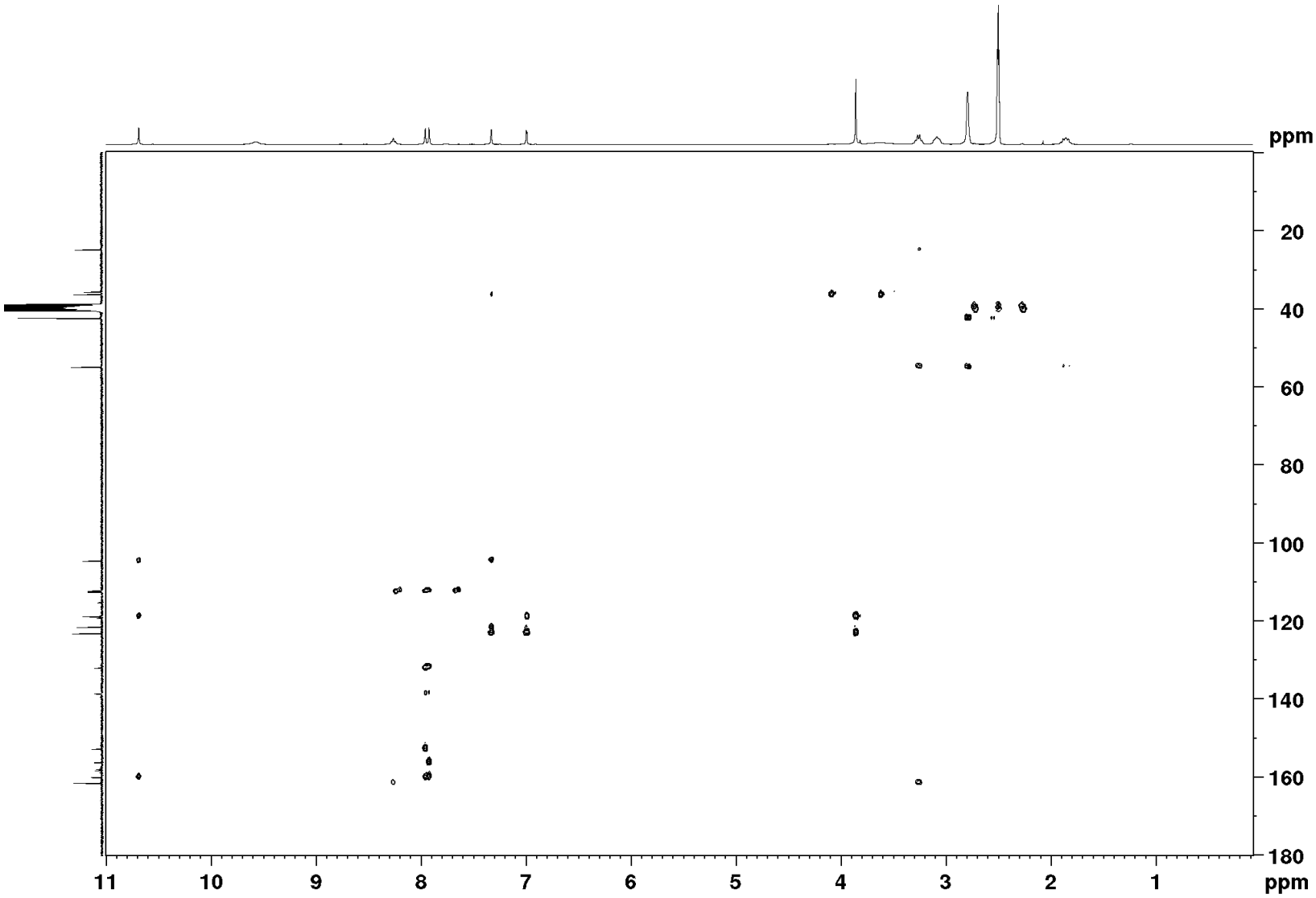



***Figure S5**: HMBC-NMR spectrum of trans-$F_4$Azo-$(PyDp)_2$ (**2**).*

***4-(4-(4-((5-((3-(dimethylamino)propyl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)carbamoyl)-2,6-difluorostyryl)-3,5-difluorobenzamido)-1-methyl-1H-pyrrole-2-carboxylic acid (19)*:** Compound **6**, (16.2 mg, 0.06 mmol) and Pd/C 5% dry (20.0 mg, 123% *w/w*) was suspended in THF (3.0 mL) and stirred under $H_2$ atmosphere for 2 h. The reaction mixture was filtered over celite and the solvent removed under reduced pressure. *$F_4$*Azo-diacid (**5**, 50.0 mg, 0.15 mmol) and HATU (33.5 mg, 0.09 mmol) were dissolved in THF (10 mL) and DIPEA (85.0 µL, 0.49 mmol) added. The reaction mixture was stirred for 50 min before the reduced pyrrole **8** dissolved in THF (1.50 mL) was added. After stirring for 1.5 h, the solvent was removed and the crude purified by preparative HPLC (5 - 60% over 40 min) to yield the desired product as its TFA salt (**19**, 19.7 mg, 0.03 mmol, 47%) as orange solid. ***$t_R$***=24.2 min (*cis*), 24.6 min (*trans*) (5% to 75% B in 30 min); **HRMS-ESI$^+$ (*m/z*):** $[M]^+$ calcd for $C_{25}H_{24}F_4N_6O_4H$, 549.1868; found, 549.1883.

***E*-isomer:**

**δH(500 MHz; [D6]DMSO; $Me_4Si$)** 13.95 (1 H, s, COOH), 10.68 (1 H, s, NH-3), 9.48 (1 H, br s, $NH^+$-12), 8.27 (1 H, t, $^3J_{HH}$ 5.8, NH-7), 7.94 (1 H, s, H-1 / H-2), 7.92 (1 H, s, H-1 / H-2), 7.83 (1 H, s, H-1b / H-2b), 7.81 (1 H, s, H-1b / H-2b), 7.32 (1 H, d, $^4J_{HH}$ 1.9, H-4), 6.99 (1 H, d, $^4J_{HH}$ 1.9, H-5), 3.85 (3 H, s, H-6), 3.28 – 3.22 (2 H, m, H-8), 3.11 – 3.06 (2 H, m, H-10), 2.79 (6 H, s, H-11 and H-11'), 1.88 – 1.81 (2 H, m, H-9); **δC (125 MHz; [D6]DMSO; $Me_4Si$)** 164.5 (COOH), 161.4 (CO), 159.9 (CO), 155.5 ($C_q$), 155.3 ($C_q$), 153.5 ($C_q$), 153.3 ($C_q$), 138.7 ($C_q$), 134.9 ($C_q$), 133.2 ($C_q$), 131.8 ($C_q$), 123.0 ($C_q$), 121.4 ($C_q$), 118.7 (CH), 114.1 (CH), 113.9 (CH), 112.4 (CH), 112.3 (CH), 104.4 (CH), 54.7 ($CH_2$), 42.2 (2C, $CH_3$), 36.2 ($CH_3$), 35.4 ($CH_2$), 24.7 ($CH_2$).

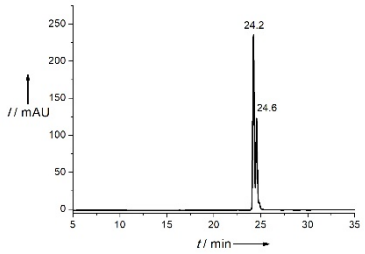



**Figure S6:** HPLC chromatogram of *$F_4$*Azo-(PyDp)(COOH) (**19**).

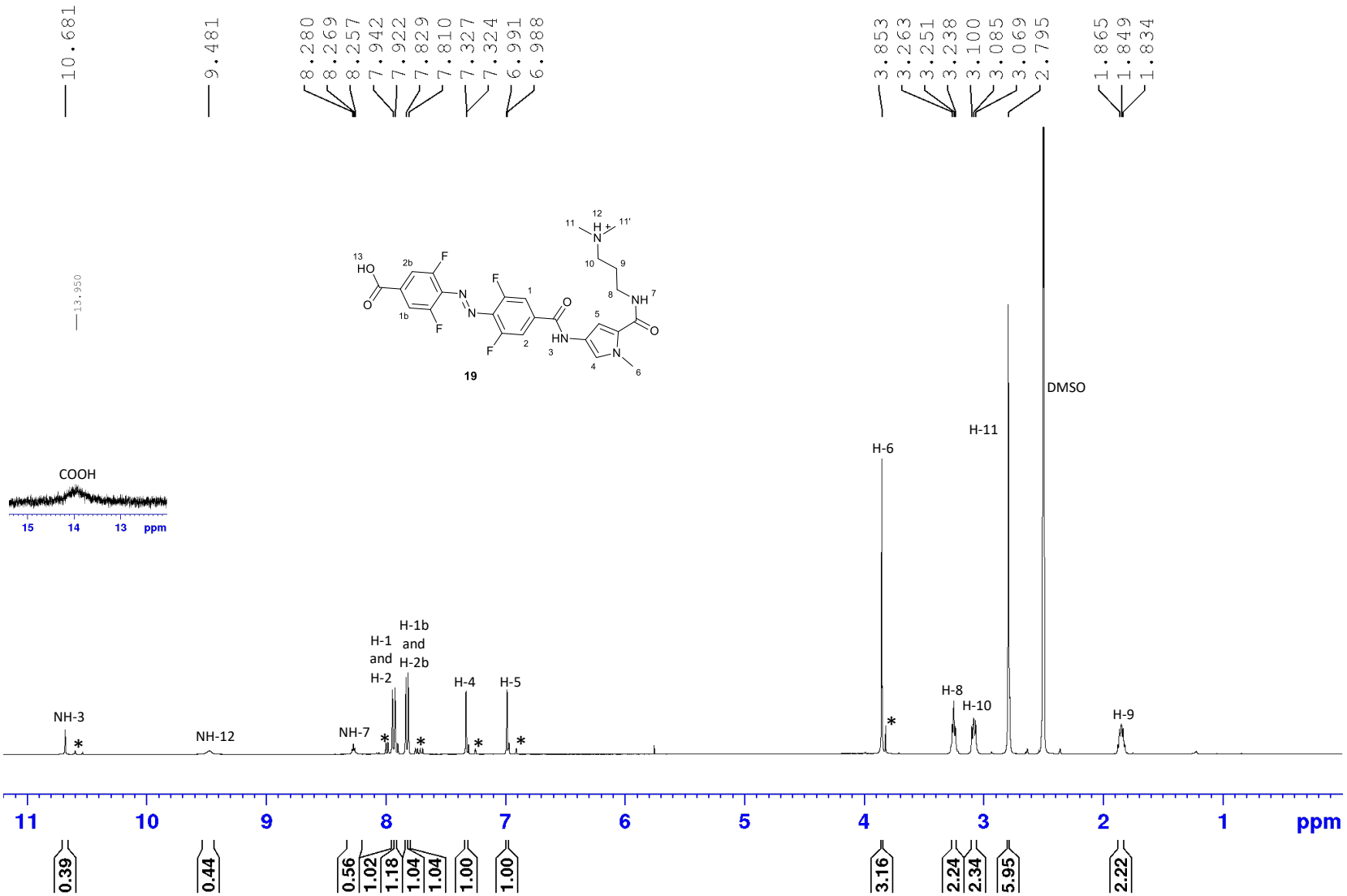



***Figure S7**: [1]H-NMR spectrum of trans-$F_4$Azo-(PyDp)(COOH) (**19**). The inset shows the signal of the acid.*

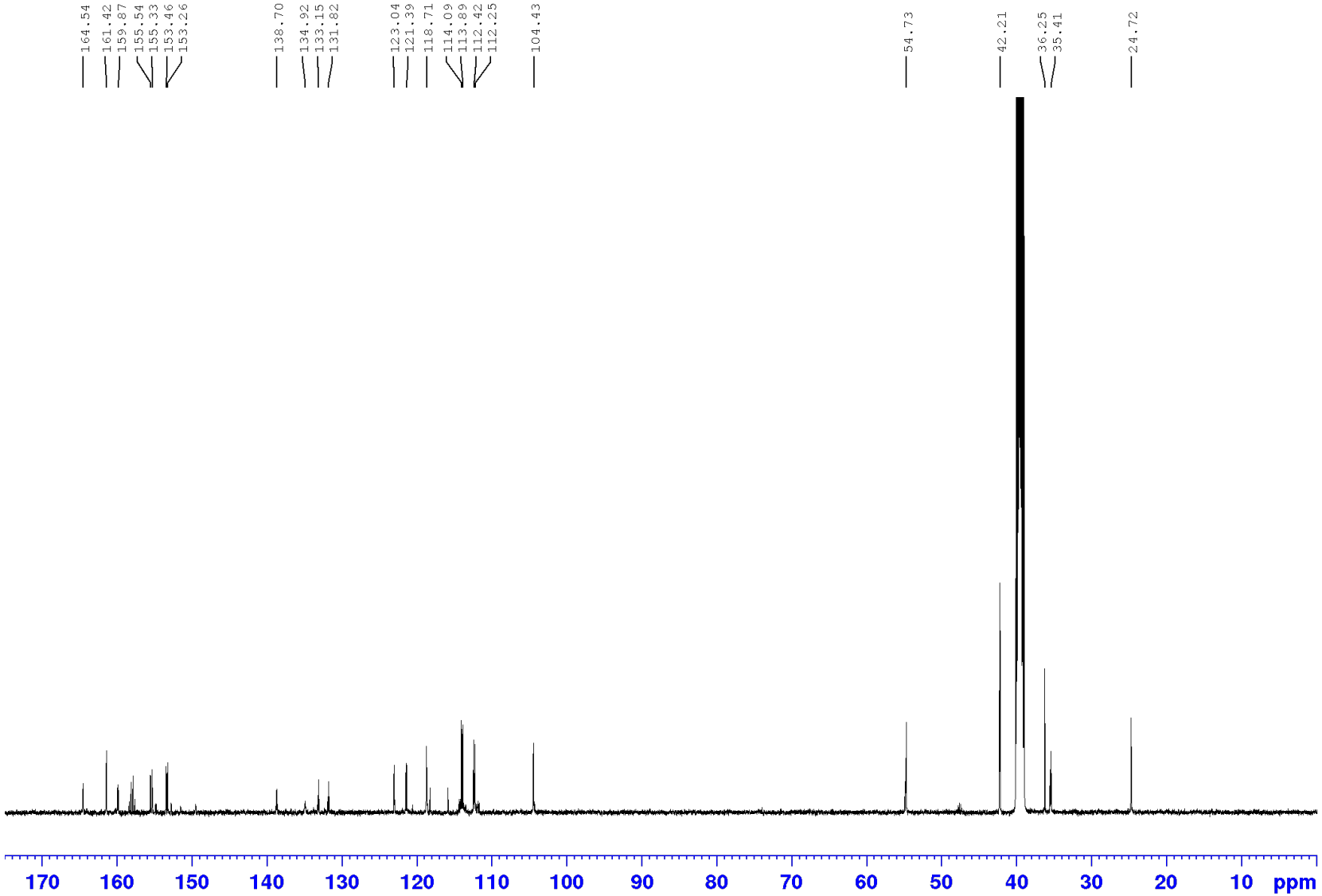



***Figure S8**: $^{13}$C-NMR spectrum of trans- $F_4$Azo-(PyDp)(COOH) (**19**).*

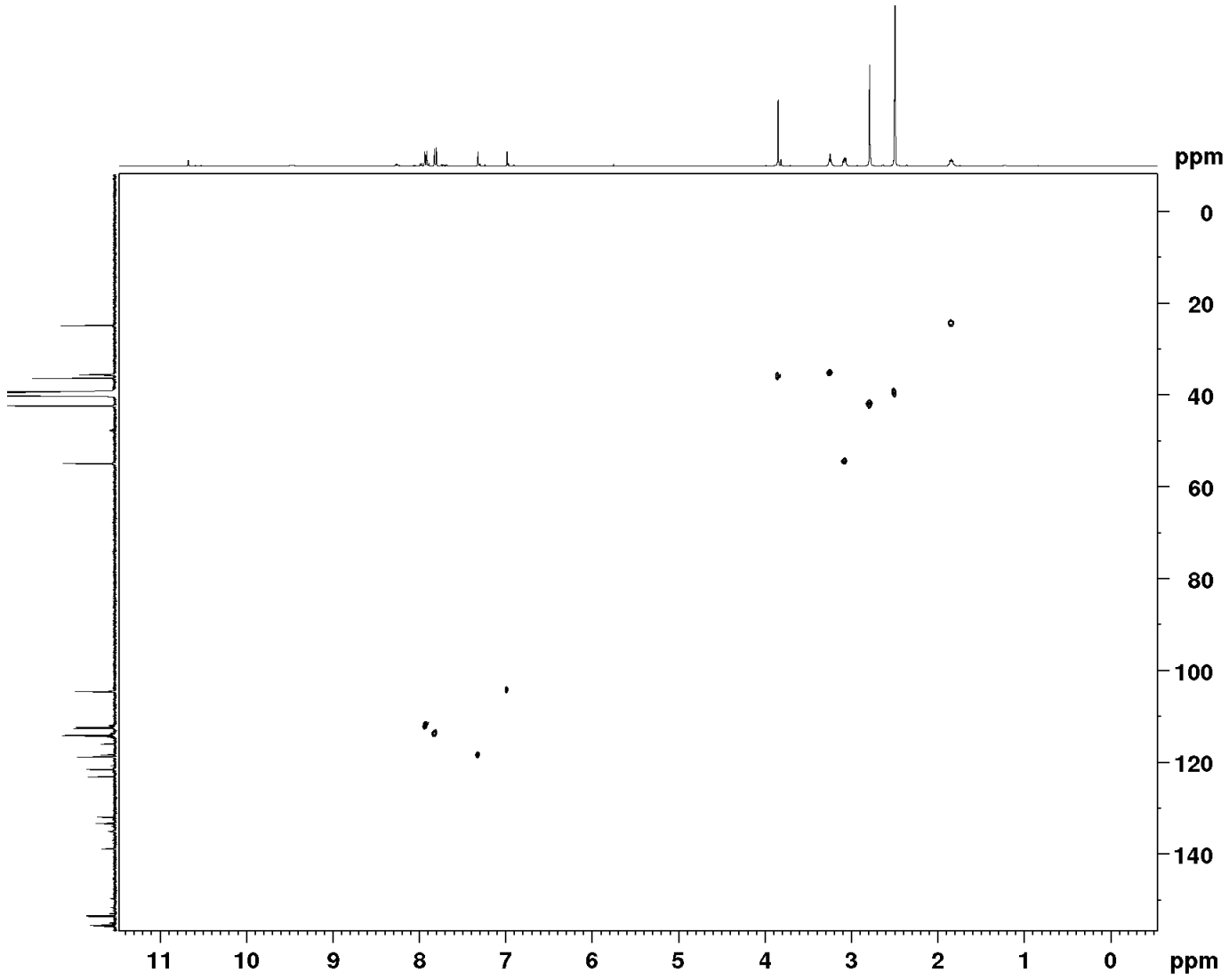



***Figure S9**: HSQC-NMR spectrum of trans- $F_4$Azo-(PyDp)(COOH) (**19**).*

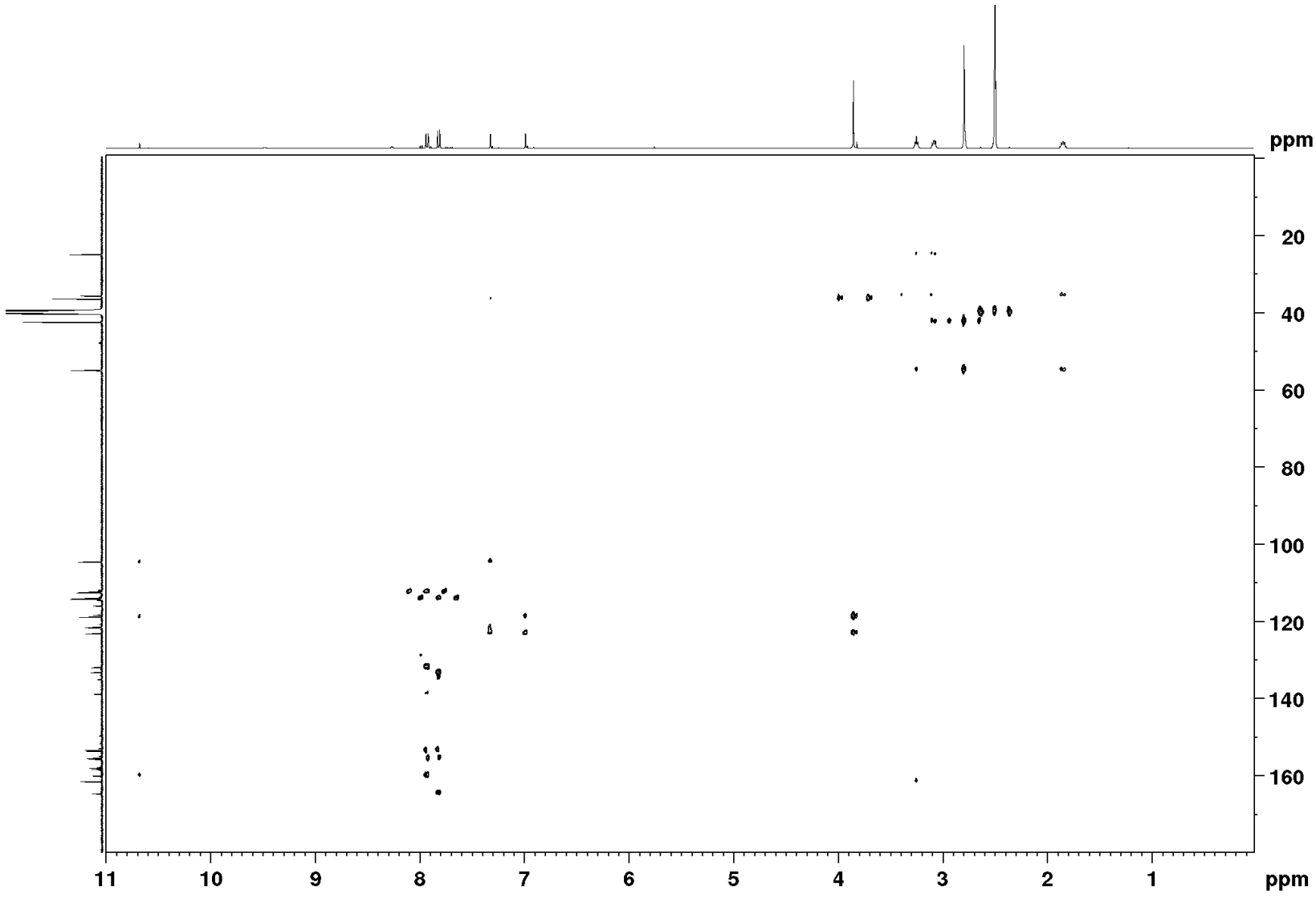



***Figure S10**: HMBC-NMR spectrum of trans- $F_4$Azo-(PyDp)(COOH) (**19**).*

***(E)-3-(4-(4-((2,6-difluoro-4-((5-(methoxycarbonyl)-1-methyl-1H-pyrrol-3-yl)carbamoyl)phenyl)diazenyl)-3,5-difluorobenzamido)-1-methyl-1H-pyrrole-2-carboxamido)-N,N-dimethylpropan-1-aminium (3)*:** Compound **7**, (4.40 mg, 24.1 µmol) and Pd/C 5% dry (6.00 mg, 136% *w/w*) was suspended in THF (3.0 mL) and stirred under $H_2$ atmosphere for 2 h. The reaction mixture was filtered over celite and the solvent removed under reduced pressure. *$F_4$*Azo-(PyDp)(COOH) (**19**, 12.0 mg, 21.9 µmol) and HATU (9.16 mg, 24.1 µmol) were dissolved in THF (400 µL) and DIPEA (12.6 µL, 72.3 µmol) added. The reaction mixture was stirred for 4 h before the reduced pyrrole **7** dissolved in THF (600 µL) was added. After stirring for 2.5 h, the solvent was removed and the crude purified by preparative HPLC (5 - 75% over 30 min) to yield the desired product as its TFA salt (**3**, 5.40 mg, 6.76 µmol, 31%) as orange solid. ***$t_R$***=27.9 min (5% to 75% B in 30 min); **HRMS-ESI$^+$ (*m/z*):** $[M]^+$ calcd for $C_{32}H_{32}F_4N_8O_5H$, 685.2505; found, 685.2532.

***E*-isomer:**

**δH(500 MHz; [D6]DMSO; $Me_4Si$)** 10.66 (1 H, s, NH-3 / NH-3b), 10.63 (1 H, s, NH-3 / NH-3b), 9.24 (1 H, br s, $NH^+$-13), 8.25 (1 H, t, $^3J_{HH}$ 5.7, NH-8), 7.94 (2 H, s, H-1 / H-2 / H-1b / H-2b), 7.91 (2 H, s, H-1 / H-2/ H-1b / H-2b), 7.56 (1 H, d, $^4J_{HH}$ 1.8, H-4b), 7.31 (1 H, d, $^4J_{HH}$ 1.8, H-4), 7.00 (1 H, d, $^4J_{HH}$ 1.8, H-5 / H-5b), 6.99 (1 H, d, $^4J_{HH}$ 1.8, H-5 / H-5b), 3.88 (3 H, s, H-6), 3.86 (3 H, s, H-6b), 3.76 (3 H, s, H-7), 3.30 – 3.22 (2 H, m, H-9), 3.13 – 3.03 (2 H, m, H-11), 2.80 (3 H, s, H-12 / H-12'), 2.79 (3 H, s, H-12 / H-12'), 1.91 – 1.78 (2 H, m, H-10); **δC (125 MHz; [D6]DMSO; $Me_4Si$)** 161.9 (CO), 161.2 (CO), 160.5 (CO), 160.4 (CO), 156.0 (2C, $C_q$), 153.9 (2C, $C_q$), 138.8 (2C, $C_q$), 132.4 (2C, $C_q$), 123.5 ($C_q$), 122.6 ($C_q$), 122.0 ($C_q$), 121.7 (CH), 119.6 ($C_q$), 119.2 (CH), 112.9 (2C, CH), 112.7 (2C, CH), 109.0 (CH), 104.9 (CH), 55.3 ($CH_2$), 51.6 ($CH_3$), 42.7 (2C, $CH_3$), 36.8 ($CH_3$), 36.7 ($CH_3$), 36.0 ($CH_2$), 25.2 ($CH_2$).

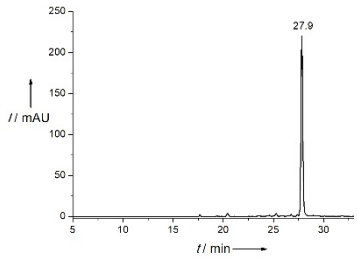



**Figure S11:** *HPLC chromatogram of $F_4$Azo-(PyDp)(PyOMe) (**3**).*

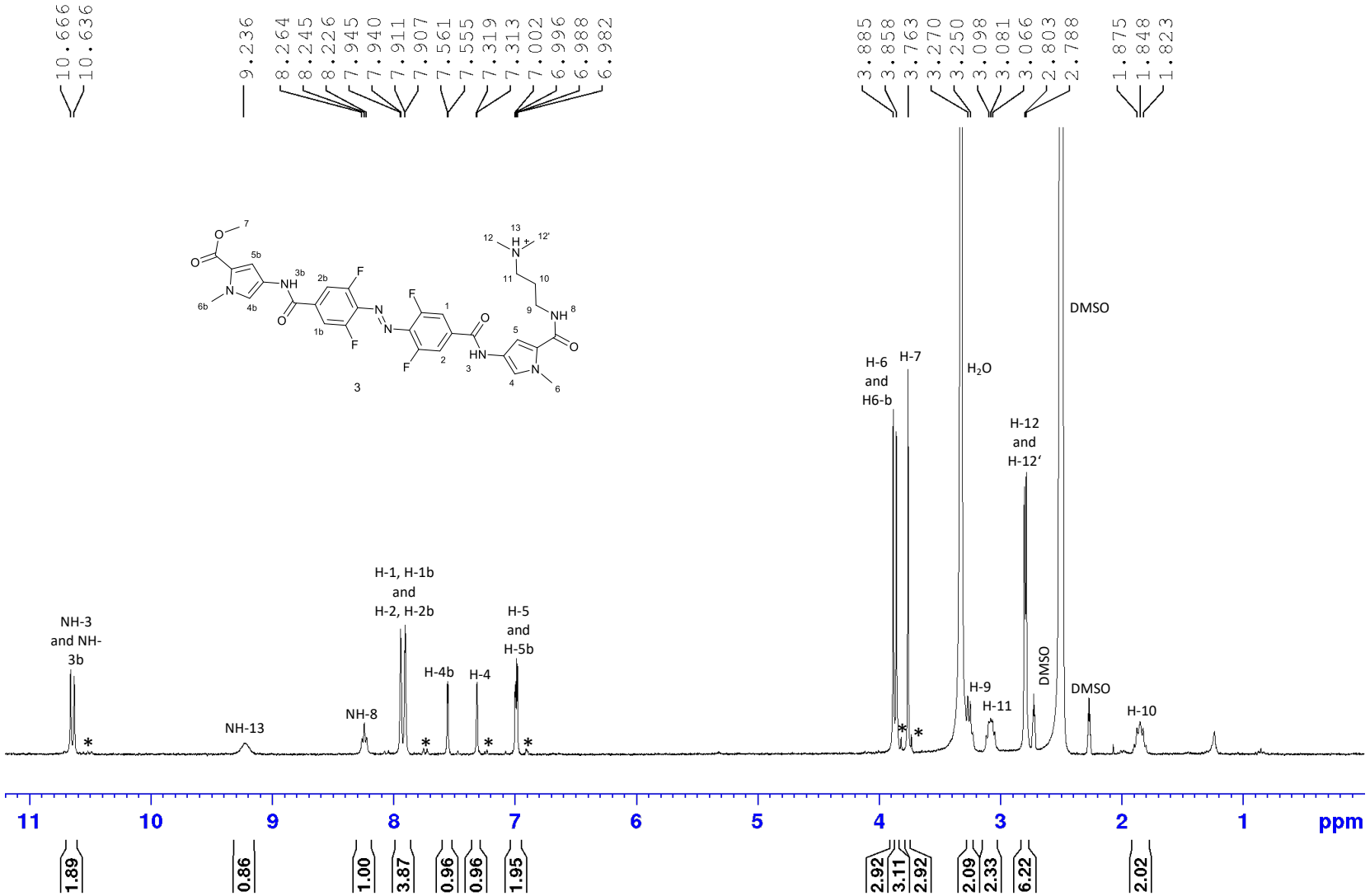



***Figure S12**: $^{1}$H-NMR spectrum of trans- $F_4$Azo-(PyDp)(PyOMe) (**3**).*

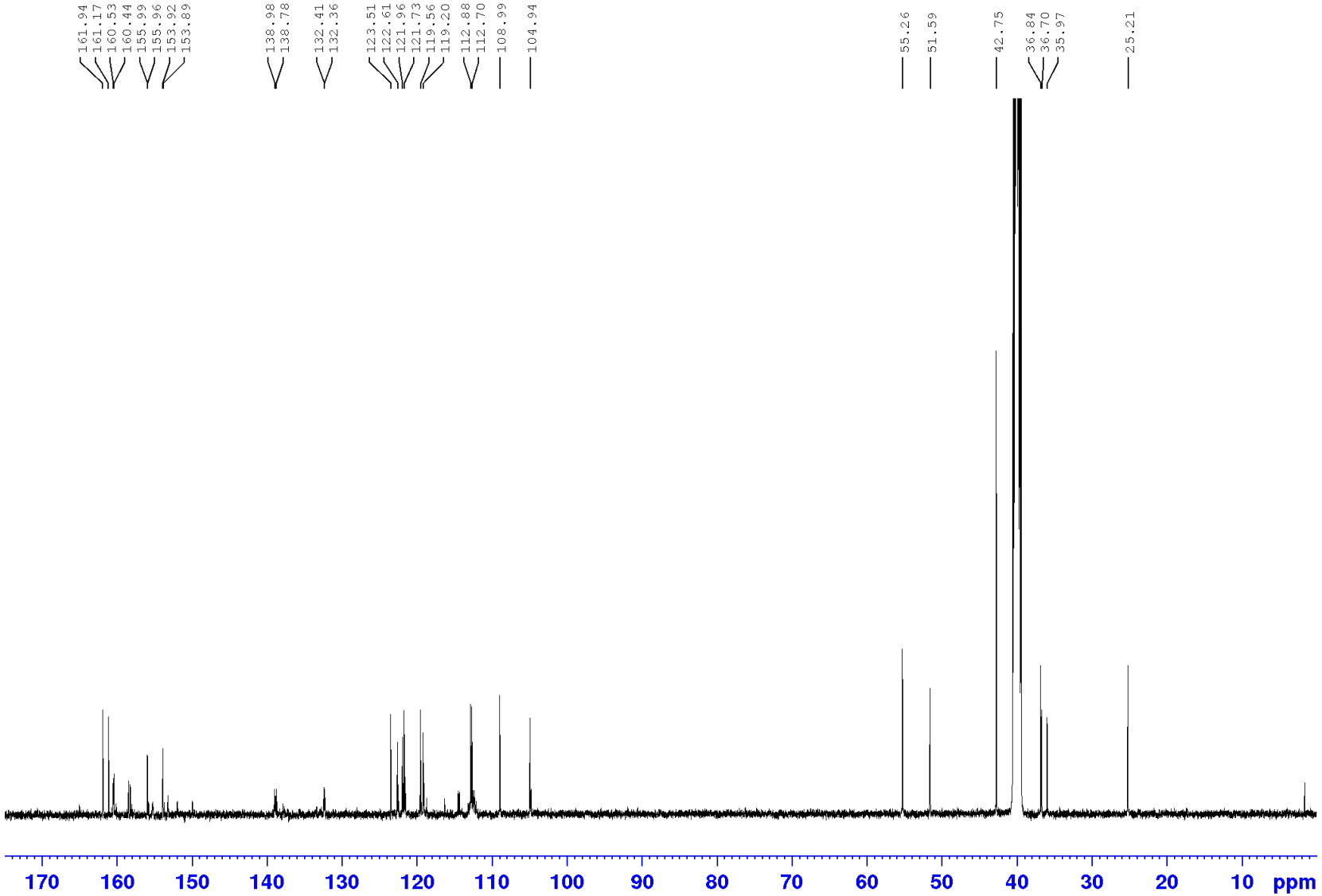



***Figure S13**: $^{13}$C-NMR spectrum of trans- trans- $F_4$Azo-(PyDp)(PyOMe) (**3**).*

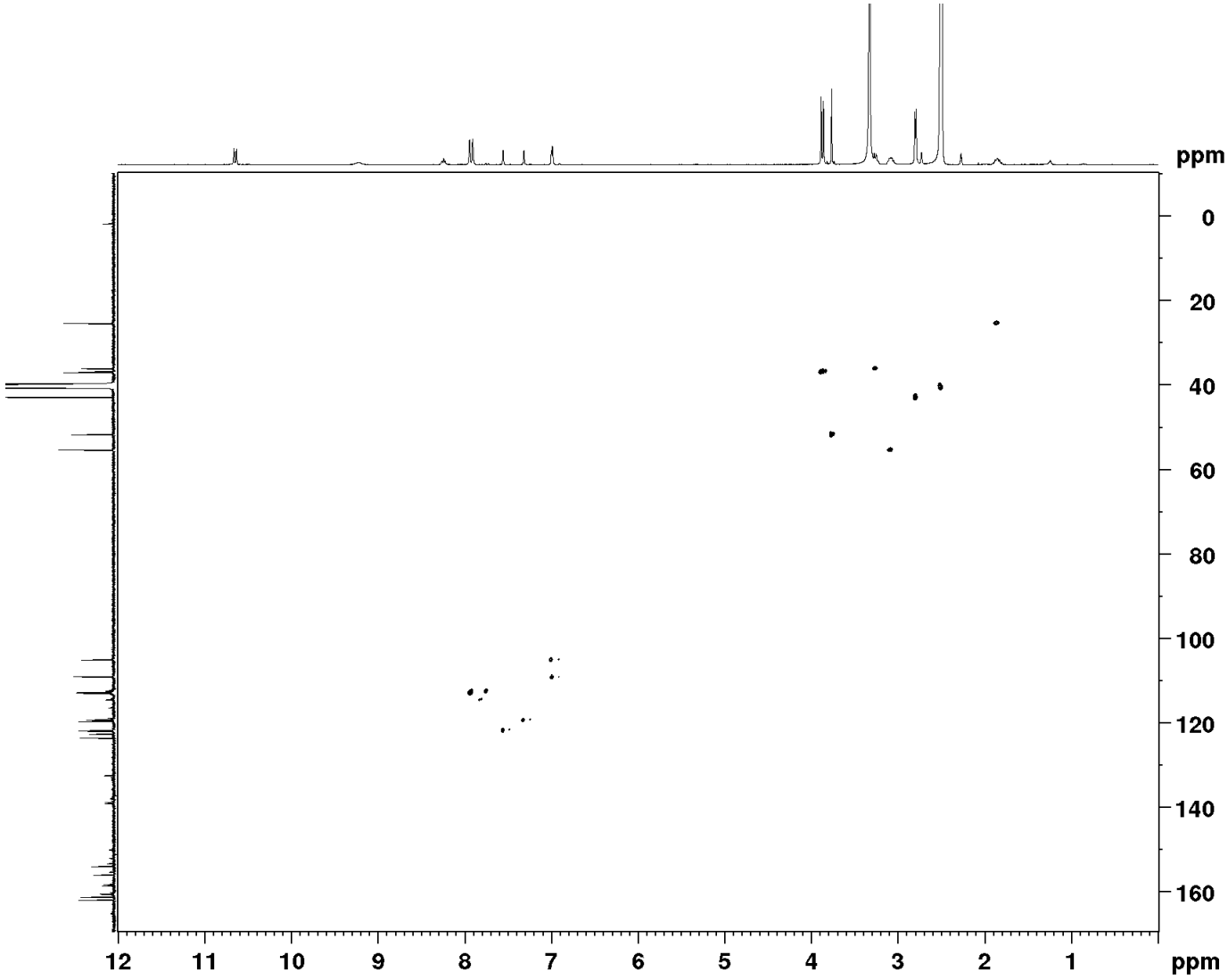



***Figure S14**: HSQC-NMR spectrum of trans- trans- $F_4$Azo-(PyDp)(PyOMe) (**3**).*

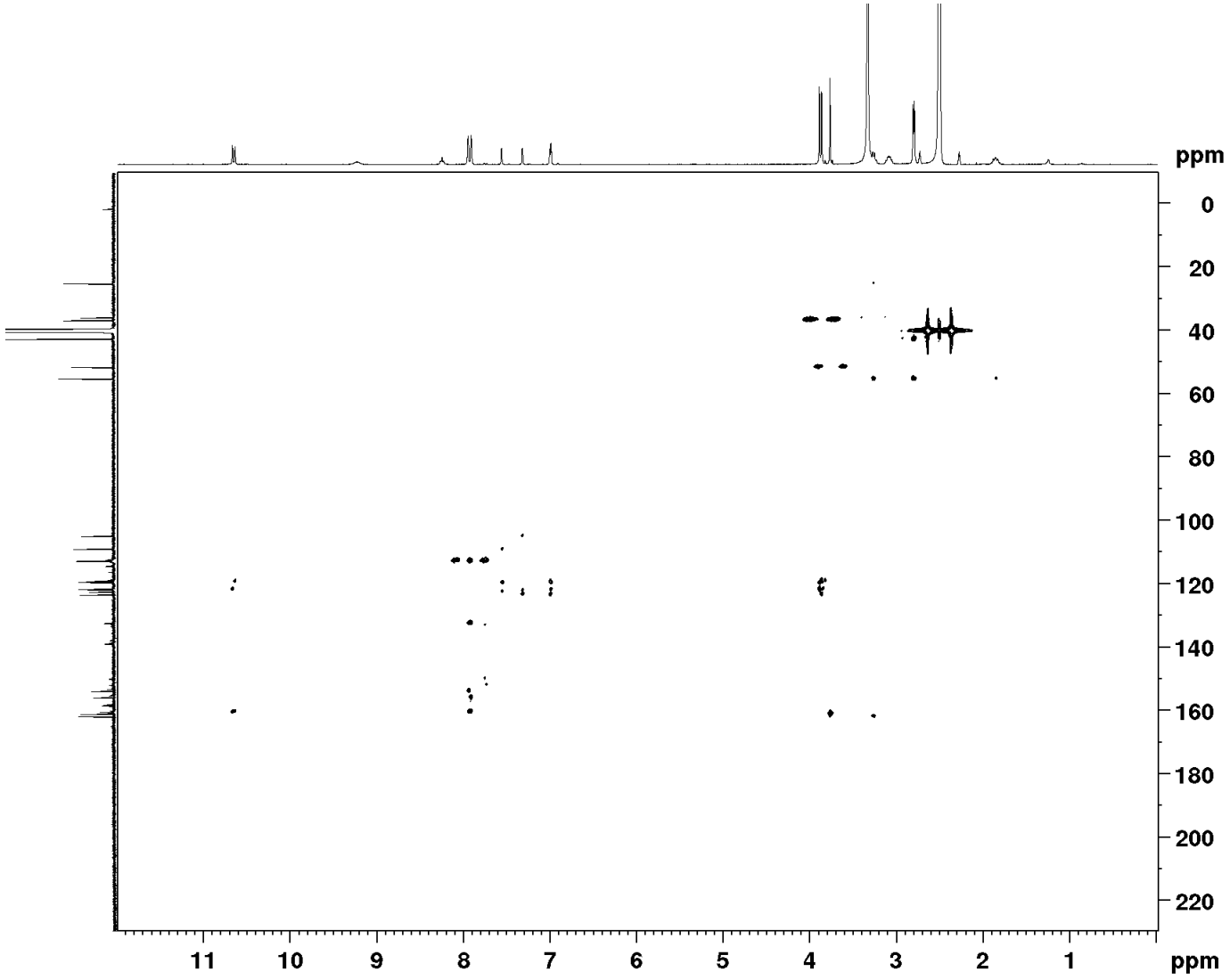



**Figure S15**: HMBC-NMR spectrum of trans- *$F_4$Azo*-(PyDp)(PyOMe) (**3**).

## Concentration Determination

For the accurate concentration determination of the compounds: $F_4$Azo-(PyDp)(PyOMe) (**3**) and $F_4$Azo-$(PyDp)_2$ (**2**) their extinction coefficient in DMSO at room temperature was calculated. A specific amount (1.00 - 2.00 mg) of the corresponding compound was weighted on a Mettler Toledo XP6 micro balance and dissolved in a known volume of DMSO. Increasing amounts of such stock solution were added into a cuvette filled with 1 mL of DMSO. The corresponding absorbance of each addition was measured on a Tecan Spark 20M at the individual maximum and without exceeding 10% of the initial volume. In all the cases the absorbance was between 0.1 and 1 AU to be in concordance with Lambert-Beer law.[vii]

$$A = \varepsilon \, l \, c \qquad \text{(S1)}$$

In this equation: A = absorbance of the solution; l = length of the cuvette; c = concentration; ε = molar extinction coefficient.
Below the UV-Vis spectrum of a 24 µM concentration of the compound $F_4$Azo-(PyDp)(PyOMe) (**3**) in DMSO (Figure S16). The lineal regression of the obtained absorbance measurements versus the concentrations of the $F_4$Azo-(PyDp)(PyOMe) allowed us to obtain the molar extinction coefficient (Figure S17). This experiment was repeated three times independently i.e. from three different stock solutions.

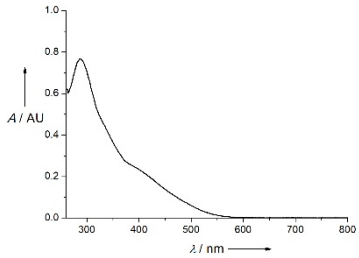



**Figure S16:** UV-Vis spectrum of a 24 µM solution of compound $F_4$Azo-(PyDp)(PyOMe) (**3**) in DMSO.

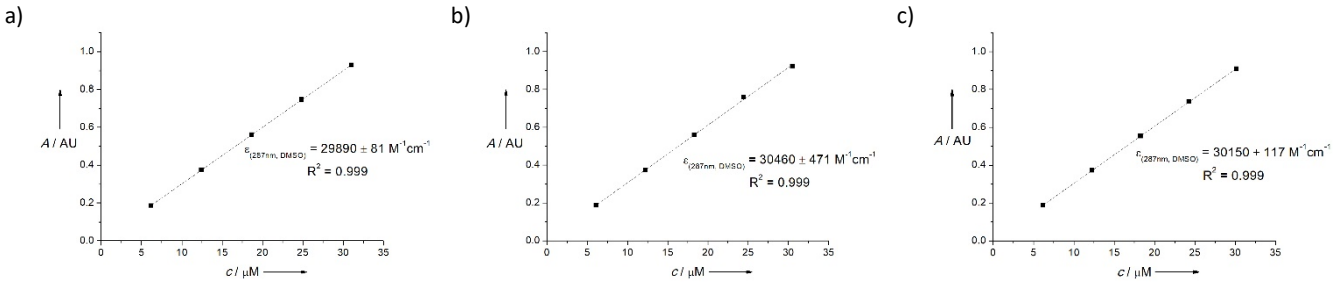



**Figure S17**: Determination of the extinction coefficient of compound $F_4$Azo-(PyDp)PyOMe) (**3**) in DMSO. The absorbance maximum at 287 nm was plotted against the $F_4$Azo-(PyDp)(PyOMe) concentration. Three independent measurements are shown with the calculated extinction coefficient and their corresponding regression value (R2). The mean extinction coefficient and the standard deviation of $F_4$Azo-(PyDp)(PyOMe) (**3**) is 30167 ± 285 $M^{-1}cm^{-1}$.

Below the UV-Vis spectrum of a 24 µM concentration of the compound $F_4$Azo-$(PyDp)_2$ (**2**) in DMSO (Figure S18). The lineal regression of the obtained absorbance measurements versus the concentrations of $F_4$Azo-$(PyDp)_2$ (**2**) allowed us to obtain the molar extinction coefficient (Figure S19). This experiment was repeated three times independently i.e. from three different stock solutions.

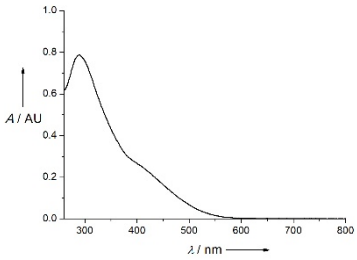



**Figure S18:** UV-Vis spectrum of a 24 µM solution of compound $F_4$Azo-(PyDp)$_2$ (**2**) in DMSO.

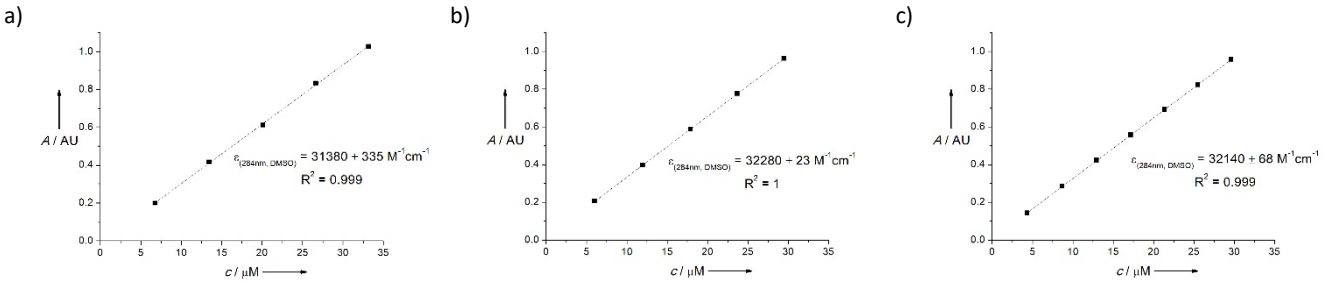



**Figure S19:** Determination of the extinction coefficient of compound $F_4$Azo-(PyDp)$_2$ (**2**) in DMSO. The absorbance maximum at 284 nm was plotted against the dimer concentration. Three independent measurements are shown with the calculated extinction coefficient and their corresponding regression value (R2). The mean extinction coefficient and the standard deviation of $F_4$Azo-(PyDp)$_2$ (**2**) is 31933 ± 484 $M^{-1}cm^{-1}$.

Table S1 summarized the obtained molar extinction coefficient for both compounds.

**Table S1:** Determined mean extinction coefficients and standard deviations of $F_4$Azo-(PyDp)(PyOMe) (**3**) and $F_4$Azo-(PyDp)$_2$ (**2**) in DMSO at r.t.

| | $F_4$Azo-(PyDp)$_2$ in DMSO (284 nm) | $F_4$Azo-(PyDp)(PyOMe) in DMSO (287 nm) |
|---|---|---|
| mean | 31933 ± 484 $M^{-1}cm^{-1}$ | 30167 ± 285 $M^{-1}cm^{-1}$ |

In general during the concentration determination, the absorption values were subtracted against the corresponding buffer solution. For thiazole orange, we used the extinction coefficient from the literature in DMSO: $\varepsilon_{503nm}$ = 63000 $M^{-1}cm^{-1}$[viii].
For the concentration determination of the hairpin DNAs, the purchased lyophilized DNA was dissolved in Milli-Q water, heated to 95°C for 10 min and slowly cooled down to room temperature. Their molar extinction coefficients were determined by using the following formula:[ix]

$$\varepsilon_{260nm} = \{(8.8 * \#T) + (7.3 * \#C) + (11.7 * \#G) + (15.4 * \#A)\} * \ 0.8 * \ 10^3 \ M^{-1}cm^{-1} \quad \text{(S2)}$$

In the formula # = number of nucleobases determined throughout the DNA sequence, T = thymine, C = cytosine, G = guanine, A = adenine. Table S2 summarizes the sequences and the determined extinction coefficients of the hairpin DNAs.

**Table S2.** Binding sites, sequences and extinction coefficients of DNA hairpins, which were used for fluorescence titrations experiments.

| DNA | binding site | sequence (5' – 3') | extinction coefficient at 260 nm |
|---|---|---|---|
| $dsDNA_{hAT}$ | *ATTA* | GGCG*ATTA*CAGCTTTTTGCTGTAATCGCC | 238400 $M^{-1}$ $cm^{-1}$ |
| $dsDNA_{hGC}$ | *GGCCC* | GGCA*GGCCC*AGCTTTTTGCTGGGCCTGCC | 225920 $M^{-1}$ $cm^{-1}$ |

The nucleic acid concentration after the PCR was determined on a NanoDrop 2000 spectrometer (Thermo Scientific) by measuring the absorbance at 260 nm and using the extinction coefficient in water for double-stranded DNA (50 ng*cm/µL).[x] $dsDNA_{CT}$ was purchased as a solution of 15.4 mM concentration.
The concentration of the nucleosomes in 10 mM Tris 1 mM EDTA pH 8.0 and 50 mM NaCl were also determined on the NanoDrop using the extinction coefficient from the literature: $\varepsilon_{260nm}$ = 1880000 $M^{-1}cm^{-1}$.[xi]

## Photoisomerization of $F_4$Azo-$(PyDp)_2$ (2) and $F_4$Azo-(PyDp)(PyOMe) (3)

A 405 nm LED lamp (Roschwege Star UV 405-03-00-00, 405 nm, 700 mA, 3.5 V, $P_{optisch}$ = 670 mW) was used for the irradiation at 405 nm and a 520 nm LED lamp (Roschwege Star LSC-G 520 nm, 1A, 3.8 V, 87 Im, 130°) was used for irradiation at 520 nm. For the irradiation at 520 nm a filter (FGL515 – Ø25 mm OG515 Colored Galss Filter, 515 nm longpass, Thorlabs) was further attached to the lamp.

For the $F_4$Azo-derivatives, the *trans*-isomer is the thermodynamically most stable form,[iii] which was also yielded from the synthesis as the major product. The photostationary states were studied by UV-Vis spectroscopy, RP-HPLC and by NMR. All photosiomerization measurements were performed in the dark.

### *UV-Vis Characterization*

For the characterization of the photoisomerization of the compound $F_4$Azo-(PyDp)(PyOMe) (**3**) via UV-Vis measurements, a solution of 20 µM of **3** in 10 mM Tris pH 7.6, 50 mM KCl and DMSO (98:2) (total volume 1 mL) was irradiated for 1 min at 405 nm to ensure complete conversion to the *trans* state. Afterwards, to obtain the *cis* isomer such solution was irradiated at 520 nm at different time intervals: 10 s / 30 s / 1 min / 2 min / 3 min / 5 min / 10 min. As seen in figure S20, after 10 min of continuous irradiation no significant spectroscopic changes could be observed. Afterwards this solution was irradiated at the time intervals: 5 s / 10 s / 30 s / 1 min / 1.5 min / 2 min / 2.5 min / 3 min at 405 nm, which brought the characteristic *trans*-isomer spectrum (Figure S20B). No significant spectroscopic changes were observed after 3 min. The represented data are the mean values calculated from three independent measurements.

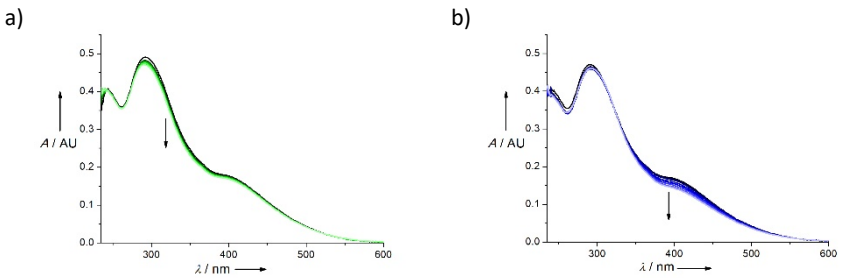



**Figure S20**: *UV-Vis spectra of 20 µM of $F_4$Azo-(PyDp)(PyOMe) (**3**) in 10 mM Tris, pH 7.6 50 mM KCl and DMSO (98:2). A) 1 min irradiation at 405 nm followed by successive time interval (10 s / 30 s / 1 min / 2 min/ 3 min / 5 min / 10 min) irradiation at 520 nm. B) Final irradiation conditions of A, followed by successive time interval (5 s / 10 s / 30 s / 1 min / 1.5 min / 2 min / 2.5 min / 3 min) irradiation at 405 nm.*

We further recorded DMSO subtracted absorbance spectrum of: a) a 40 µM solution of $O_2$N-Py-Dp (**8**) in 10 mM Tris pH 7.6, 50 mM KCl and DMSO (98:2); b) 20 µM solution of $F_4$Azo-$(COOH)_2$ (**5**) in the *trans* and the *cis*-state in 10 mM Tris 50 mM pH 7.6, KCl and DMSO (98:2) and c) the combined solutions in the *trans* and the *cis*-state. Figure S21 shows the described spectra.

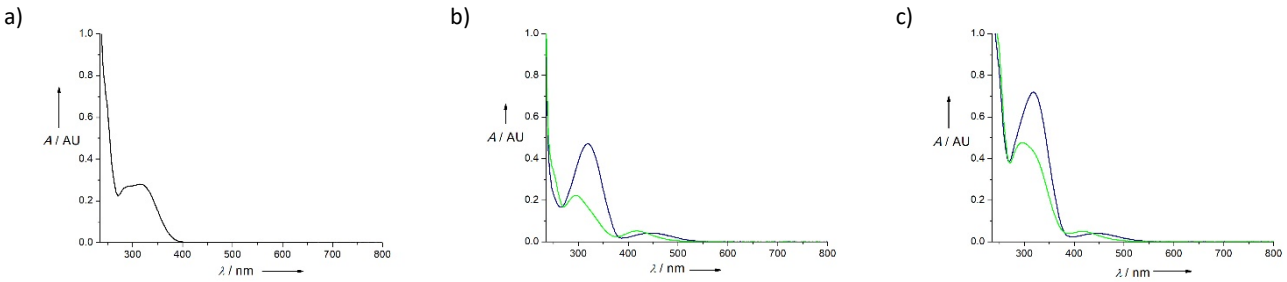



**Figure S21:** *A) UV-Vis spectra of 40 µM $O_2$N-Py-Dp (**8**) in 10 mM Tris pH 7.6, 50 mM KCl and DMSO (98:2). B) UV-Vis spectra of 20 µM $F_4$Azo-$(COOH)_2$ (**5**) in 10 mM Tris pH 7.6 , 50 mM KCl and DMSO (98:2). The green spectra shows the compound in the cis-state, which was achieved by irradiating of the sample for 2 min at 520 nm. The blue spectra shows the compound in the trans-state, which was achieved by irradiating the sample for 10 s at 405 nm C) UV-Vis spectra of a mixture of 40 µM $O_2$N-Py-Dp (**8**) and 20 µM $F_4$Azo-$(COOH)_2$ (**5**) in 10 mM Tris pH 7.6, 50 mM KCl and DMSO (98:2). The green spectra shows the compound in the cis-state, which was achieved by irradiating the sample for 2 min at 520 nm. The blue spectra shows the compound in the trans-state, which was achieved by irradiating the sample for 10 s at 405 nm.*

We further recorded the spectra of the *trans*- and the *cis*-isomer of a 20 µM solution of $F_4$Azo-$(PyDp)_2$ (**2**) in DMSO under the same conditions, which spectra is shown in figure S22.

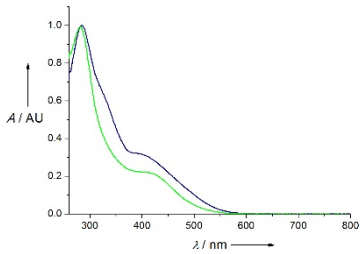



**Figure S22:** UV-Vis spectra of 20 µM of $F_4$Azo-$(PyDp)_2$ (**2**) in DMSO. The blue spectra shows the spectra of the *trans*-isomer, which was achieved by 1 min irradiation at 405 nm. The green spectra shows the spectra of the *cis*-isomer, which was achieved by irradiation at 520 nm for 2 min.

The procedure of the absorbance titrations was: addition of increasing amounts of a 2.73 mM $F_4$Azo-$(PyDp)_2$ solution in DMSO to solution of 12.5 µM ds$DNA_{CT}$ in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl in the corresponding cuvette. The stock solution of $F_4$Azo-$(PyDp)_2$ was irradiated for 10 s at 405 nm to measure the *trans*-derivative or 2 min at 520 nm to measure the *cis*-derivative.

Solutions were kept in the dark and the measurement performed in the absence of light. Small aliquots of the compound stocks were stepwise added and the fluorescence spectra recorded after an incubation time of 3 min. The corresponding spectra are buffer subtracted and show the mean out of three measurements.

## *RP-HPLC Characterization*

For the characterization of the photoisomerization of the compounds via RP-HPLC measurements, a solution of 50 µM of each compound in 10 mM Tris pH 7.4, 10 mM NaCl and DMSO (95:5) was irradiated for 10 s at 405 nm to obtain the *trans*-isomer and for 2 min at 520 nm to obtain the *cis*-isomer and 30 µL were injected into the RP-HPLC. The photostationary states reached upon irradiation were determined by integration of the peak areas in the HPLC chromatogram at 287 nm (isosbestic point). In the case of $F_4$Azo-$(PyDp)_2$ (**2**) it was possible to detect both isomers by RP-HPLC, in the case of $F_4$Azo-(PyDp)(PyOMe) (**3**) it was not possible to separate both isomers by RP-HPLC and analyze their photoisomerization. The represented data shows a representative one of three measurements from independent experiments. Figure S23 shows the corresponding RP-HPLC chromatograms with their *trans*/*cis*-ratio after irradiation of the samples.

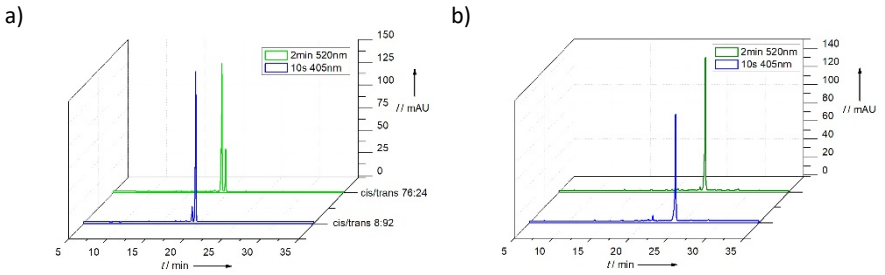



**Figure S23**: *RP-HPLC chromatogram of A) $F_4$Azo-$(PyDp)_2$ (**2**) and B) $F_4$Azo-(PyDp)(PyOMe) (**3**) after irradiation at 405 nm for 10 s and 520 nm for 2 min and their corresponding trans/cis-ratio.*

## *NMR Characterization*

For the characterization of the photoisomerization of the compounds via NMR, a solution of 2 mg of each compound was dissolved in $DMSO_{d6}$ to give a 4 mM solution and their $^1$H-NMR spectra was measured in a brown NMR tube (Rotilabo-NMR tube, Carl Roth) to get the spectrum of the thermodynamically more stable *trans*-isomer. To get the spectrum of the *cis*-isomer the solution was irradiated for 2 min at 520 nm, transferred into the Rotilabo-NMR tube and its $^1$H-NMR spectra was measured. In the case of $F_4$Azo-(PyDp)(PyOMe) (**3**) another spectrum after irradiation for 4 min at 520 nm was recorded, to ensure the maximum conversion after 2 min of irradiation, which was determined for $F_4$Azo-$(PyDp)_2$ (**2**) as sufficient. The *trans*/*cis*-ratio was calculated by integration of the proton signals of the pyrroles and the aromatic protons of the tetra-ortho-fluorazobenzenes. The represented data shows a representative one of three measurements from independent experiments.

## DNA Binding Experiments

DNA binding affinities were determined via fluorescence intercalation displacement (FID) titrations. To a solution of hairpin DNA, thiazole orange (TO) was added yielding in a fluorescence increase upon binding to the DNA. Addition of our compounds led to a decrease of fluorescence due to the displacement of the fluorescent TO. The affinity of TO to the respective hairpin DNA, allows the determination of the binding affinity of our molecules through the fluorescence decrease measurements.[xii] We decided to use TO instead of EtBr, as it gives a much higher fluorescence increase upon DNA binding and therefore, allowed to use smaller slits sizes in our FID assay. This results in less light exposure of our samples and a lower probability of back-isomerization during the measurement.[xiii]

To determine the binding affinity of TO to the hairpin DNAs, titrations were performed in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl (total volume 1 mL) at a constant TO concentration of 1 µM. Small aliquots of DNA stocks (50 µM and 500 µM) were stepwise added and the fluorescence spectra recorded after an incubation time of 3 min. The corresponding spectra are buffer subtracted and show the mean out of three independent measurements. The maximum at 530 nm was plotted versus the added amount of DNA. Represented data are mean values calculated from three independent experiments. The binding affinity was calculated using the program HypSpec using the non-corrected measured spectra.

The following figures show the recorded mean fluorescence spectra of TO with $dsDNA_{hAT}$ and $dsDNA_{hGC}$ and its maximum plotted against the added amount of DNA.

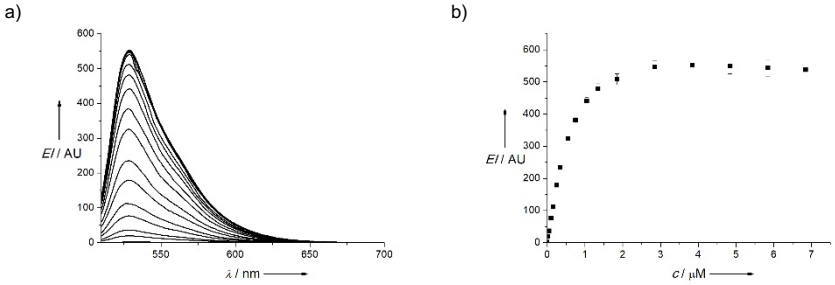



**Figure S24**: *A) Mean fluorescence spectra of a 1 µM solution of TO in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl with increasing amounts of $dsDNA_{hAT}$. B) Fluorescence intensity maximum at 530 nm plotted against the concentration of added DNA. Represented data are mean values and their standard deviation calculated from three independent experiments.*

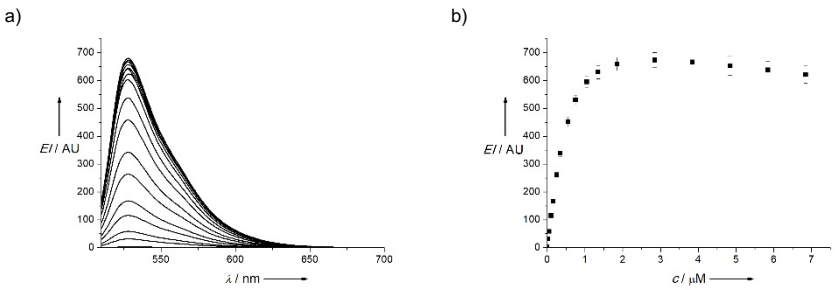



**Figure S25**: *A) Mean fluorescence spectra of a 1 µM solution of TO in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl with increasing amounts of $dsDNA_{hGC}$. B) Fluorescence intensity maximum at 530 nm plotted against the concentration of added DNA. Represented data are mean values and their standard deviation calculated from three independent experiments.*

Using the program HypSpec and setting the number of reagents to 2, the corresponding binding affinities were calculated out of the non-corrected measured spectra and summarized in table S5 (Section Binding Affinity Calculation by HypSpec).

Once the $K_D$ of TO to the hairpin DNAs was determined, we performed the FID titrations to determine the $K_D$ of the compounds. Varying from the described procedure above, the slits for the displacement titrations were excitation slit width: 3 nm; emission slit width: 3 nm. Titrations were performed in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl (total volume 1 mL) at a TO concentration of 6 µM and a hairpin concentration of 1 µM, which equals the ideal dye:bp-DNA ratio of 1:2.[xii,xiv] Compound solutions were prepared as a DMSO stock with a concentration of 250 µM and 1 mM. These stock solutions were irradiated for 10 s at 405 nm to measure the *trans*-derivative or 2 min at 520 nm to measure the *cis*-derivative. Netropsin was prepared as DMSO stocks with the same concentrations, but not irradiated. Solutions were kept in the dark and the measurement performed in the absence of light. Small aliquots of the compound stocks were stepwise added and the fluorescence spectra recorded after an incubation time of 3 min. The corresponding spectra are buffer subtracted and show the mean out of three independent measurements. The maximum at 530 nm was plotted against the added amount of compound. In the plot of the fluorescence intensity against the added compound concentration, the mean value and its standard deviation was calculated out of three independent measurements. The binding affinity was calculated using the program HypSpec and setting the number of reagents to 3, using the non-corrected measured spectra.

The following figures show the recorded mean fluorescence spectra of the FID titrations of TO and $dsDNA_{hAT}$ with *F*$_4$Azo-(PyDp)(PyOMe) (**3**) and *F*$_4$Azo-$(PyDp)_2$ (**2**) each in the *trans*- and *cis*-conformation and netropsin.

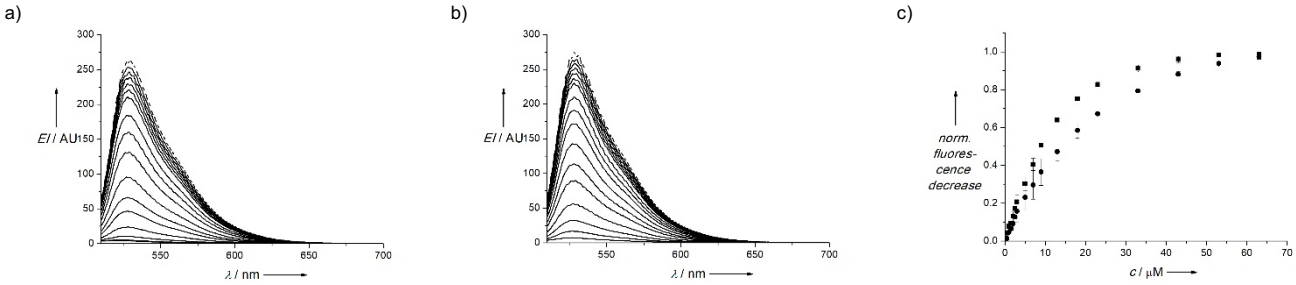



**Figure S26:** *dashed line: mean fluorescence emission spectra of a 1 µM TO and 6 µM $dsDNA_{hAT}$ solution in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl. Solid lines: mean fluorescence emission spectra of solutions of a 6 µM TO and 1 µM $dsDNA_{hAT}$ solution in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl and increasing amounts of $F_4$Azo-(PyDp)(PyOMe) (**3**) in A) trans- conformation and B) cis- conformation. C) Fluorescence intensity maximum at 530 nm plotted against the concentration of added compound. Represented data are mean values and their standard deviation calculated from three independent experiments. Squares show the plot of $F_4$Azo-(PyDp)(PyOMe) (**3**) in the trans- conformation and circles in the cis- conformation.*

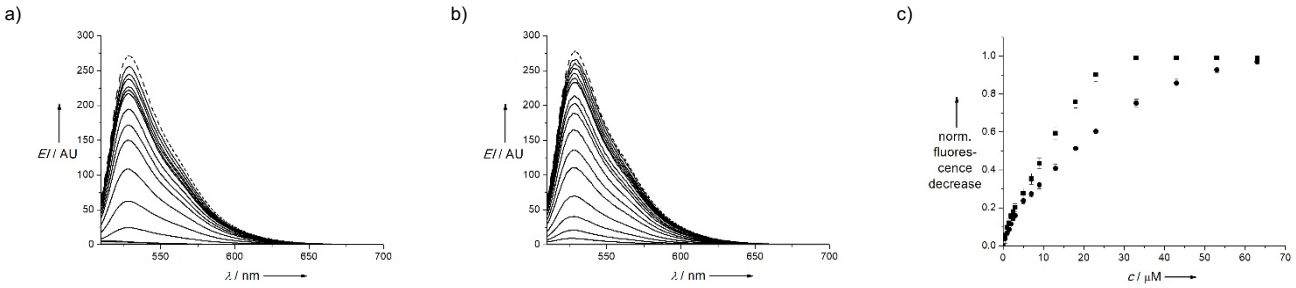



**Figure S27:** *dashed line: mean fluorescence emission spectra of a 1 µM TO and 6 µM dsDNA$_{hAT}$ solution in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl. Solid lines: mean fluorescence emission spectra of solutions of a 6 µM TO and 1 µM dsDNA$_{hAT}$ solution in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl and increasing amounts of $F_4$Azo-$(PyDp)_2$ (**2**) in A) trans- conformation and B) cis-conformation. C) Fluorescence intensity maximum at 530 nm plotted against the concentration of added compound. Represented data are mean values and their standard deviation calculated from three independent experiments. Squares show the plot of $F_4$Azo-$(PyDp)_2$ (**2**) in the trans-conformation and circles in the cis- conformation.*

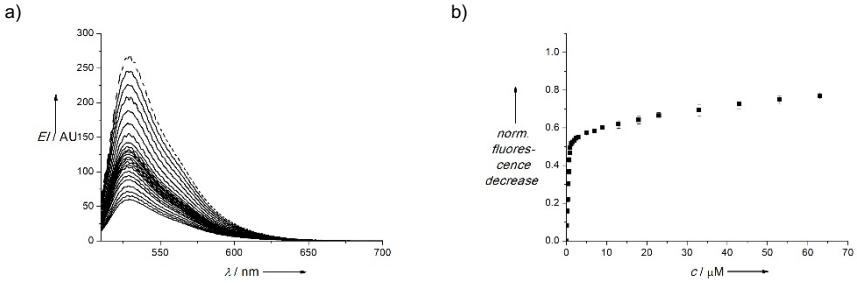



**Figure S28:** A) *dashed line: mean fluorescence emission spectra of a 1 µM TO and 6 µM dsDNA$_{hAT}$ solution in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl. Solid lines: mean fluorescence emission spectra of solutions of a 6 µM TO and 1 µM dsDNA$_{hAT}$ solution in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl and increasing amounts of netropsin. B) Fluorescence intensity maximum at 530 nm plotted against the concentration of added compound. Represented data are mean values and their standard deviation calculated from three independent experiments.*

Using the program HypSpec the corresponding binding affinities were calculated out of the non-corrected measured spectra and summarized in table S6 (Section Binding Affinity Calculation by HypSpec).

To check for the selectivity of the binder, $F_4$Azo-$(PyDp)_2$ (**2**) in the *trans*-conformation and netropsin were used in a FID titration with dsDNA$_{hGC}$. The following figures show the recorded mean fluorescence spectra.

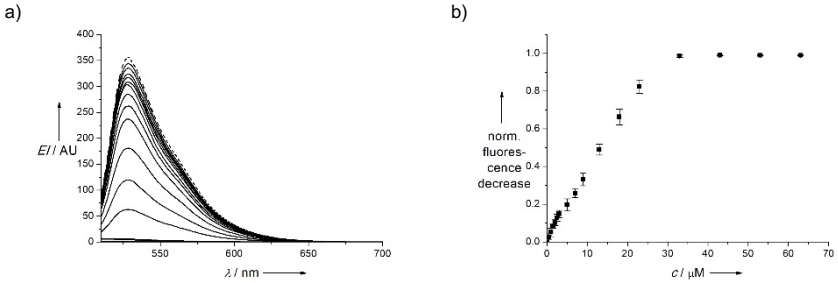



**Figure S29:** A) *dashed line: mean fluorescence emission spectra of a 1 µM TO and 6 µM dsDNA$_{hGC}$ solution in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl. Solid lines: mean fluorescence emission spectra of solutions of a 6 µM TO and 1 µM dsDNA$_{hGC}$ solution in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl and increasing amounts of trans-$F_4$Azo-$(PyDp)_2$. B) Fluorescence intensity maximum at 530 nm plotted against the concentration of added compound. Represented data are mean values and their standard deviation calculated from three independent experiments.*

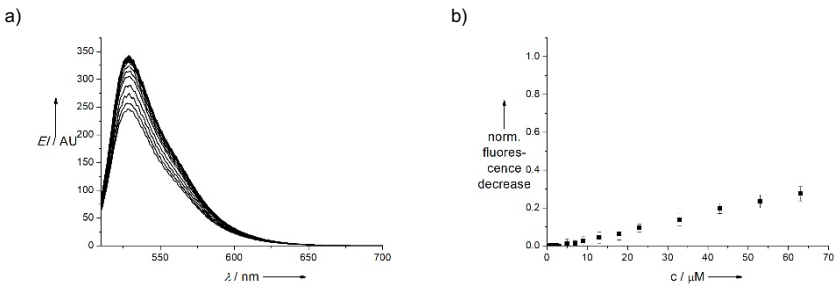



**Figure S30:** A) *dashed line: mean fluorescence emission spectra of a 1 µM TO and 6 µM dsDNA$_{hGC}$ solution in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl. Solid lines: mean fluorescence emission spectra of solutions of a 6 µM TO and 1 µM dsDNA$_{hGC}$ solution in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl and increasing amounts of netropsin. B) Fluorescence intensity maximum at 530 nm plotted against the concentration of added compound. Represented data are mean values and their standard deviation calculated from three independent experiments.*

Using the program HypSpec the corresponding binding affinities were calculated out of the non-corrected measured spectra and summarized in table S7 (Section Binding Affinity Calculation by HypSpec).

## *Complex Formation Calculation by HySS2009*

To have reliable results from the FID assays, all intercalation sites must be occupied with the minimal presence of free molecule in solution to maintain background fluorescence low and fluorescence decrease sufficiently large to give an adecuate dynamic range.[xiv] To validate whether the used conditions during the FID are appropiated, we simulated how much percentage of TO-DNA complex is formed at the initial point of our FID assays by using the software Hyperquad Simulation and Speciation, HySS2009 (Protonic Software).[xv] We analyzed *calf thymus* DNA and DNA hairpins. The simulation mode was used, the number of reagents was set to 3 (TO, DNA, analyzed compound) and protons set to be absent. The input of the program was the concentration of the species and the binding constant of the TO-DNA complex, which was set to be constant. The binding affinity of the TO-DNA complex for the hairpin DNAs was determined experimentally (see table S3) and the one for *calf thymus* DNA was $K_D$ = 3.16 µM.[xvi] The percentage of formed complex was obtained from simulated graph. Table S3 shows the percentage of the *calf thymus* DNA-TO complex at different concentrations and ratios of both. We were not able to find suitable low micromolar concentrations resulting in a complex formation >95%. For the DNA hairpins, which are classically used in FID assays[xii] we were able to reach complex formations >95% with the conditions which were later applied. The complex percentages with the hairpin DNAs are shown in table S4.

**Table S3:** Simulated *calf thymus* DNA-TO complex percentages with different concentrations and ratios of *calf thymus* DNA and TO. The given percentages were simulated by using the program HySS2009.

| DNA concentration [µM] | TO concentration [µM] | TO-DNA-complex |
|---|---|---|
| 1 | 1 | 20% |
| | 2 | 34% |
| | 5 | 42% |
| | 10 | 74% |
| | 20 | 86% |
| 5 | 1 | 11% |
| | 2 | 22% |
| | 5 | 46% |
| | 10 | 68% |
| | 20 | 83% |
| 10 | 1 | 7% |
| | 2 | 14% |
| | 5 | 34% |
| | 10 | 57% |
| | 20 | 79% |

**Table S4:** Simulated hairpin DNA-TO complex percentages with different concentrations and ratios of hairpin DNAs and TO. The given percentages were simulated by using the program HySS2009.

| DNA concentration | TO concentration [µM] | TO-DNA-complex |
|---|---|---|
| 1 µM $dsDNAh_{AT}$ | 1 | 78% |
| | 6 | >95% |
| 1 µM $dsDNAh_{GC}$ | 1 | 90% |
| | 6 | >95% |

## *Binding Affinity Calculation by HypSpec*

Binding affinities were calculated by using the software HypSpec2014 (Protonic Software).[xvii] For the calculation of the binding affinities of TO to the hairpin DNAs, the number of reagents was set to 2 (TO and DNA), assuming a 1:1 binding mode. The fluorescence spectra of TO alone under the same conditions as described above was measured and due to its low intensity (see figure S31) only the spectra of the TO-DNA-complex was set to have a fluorescence spectra. All other spectra were assumed to be none. pH readings were set to be absent. The spectra of the fluorescence measurements, including the concentration of each

reagent at each measurement point was prepared in an extra file and loaded into the program. The binding affinity of the TO-DNA-complex was refined, aiming the lowest sigma value, assuring that the molar absorbances are positive and the percentage of the formed complexes and free reagents make sense. Represented data are mean values calculated from three independent experiments. Figure S32 shows exemplary one out of the three binding affinity determination fits of HypSpec at a wavelength of the maximum of 530 nm. Table S5 summarized the determined binding affinities.

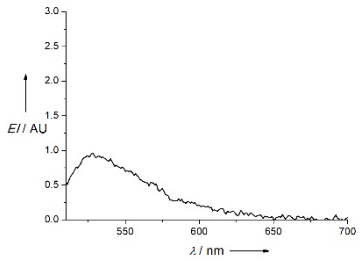



**Figure S31:** Mean fluorescence spectra of a 1 µM solution of TO in 20 mM $NaH_2PO_4$ 100 mM NaCl pH 7.4.

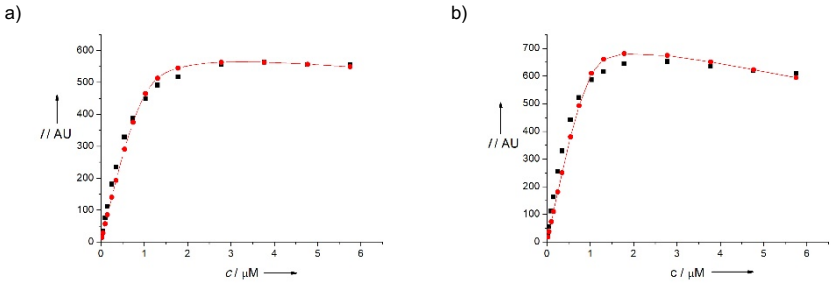



**Figure S32:** Representative plots of the binding affinity determination of TO to A) ds*DNA*$_{hAT}$ and B) ds*DNA*$_{hGC}$ at the wavelength of the maximum of 530 nm. Shown are in black the intensities experimentally determined at different concentrations of *ds*DNA and in red the corresponding fit performed by HypSpec.

**Table S5:** Determined mean $K_D$-values and standard deviations of TO to dsDNA$_{hAT}$ and dsDNA$_{hGC}$.

| | TO to dsDNA$_{hAT}$ | TO to dsDNA$_{hGC}$ |
|---|---|---|
| Mean $K_D$ value | 59.8 ± 13.1 nM | 11.6 ± 3.8 nM |

For the calculation of the binding affinities of $F_4$Azo-(PyDp)(PyOMe) (**3**) and $F_4$Azo-(PyDp)$_2$ (**2**) in the *trans*- and *cis*-conformation it was proceeded like described above, but the number of reagents was set to 3 (TO, DNA and compound **2** or **3**). Only the spectra of the TO-DNA-complex was set to have a fluorescence spectra, as the spectrum of TO alone is very low compared to the recorded ones (see fig. S321). The binding affinity of the TO-DNA complex was set to constant using the constant value, which was calculated above (table S5). The binding affinity of the binder-DNA complex was refined like described above. For the case of the netropsin (**1**) the best fit was obtained using the data up to 2 µM concentration; higher concentrations suggest different binding modes.

Represented data are mean values calculated from three independent experiments Figure S33 shows exemplary one out of the three binding affinity determination fits of HypSpec at a wavelength of the maximum of 530 nm. Table S6 and S7 summarized the determined binding affinities.

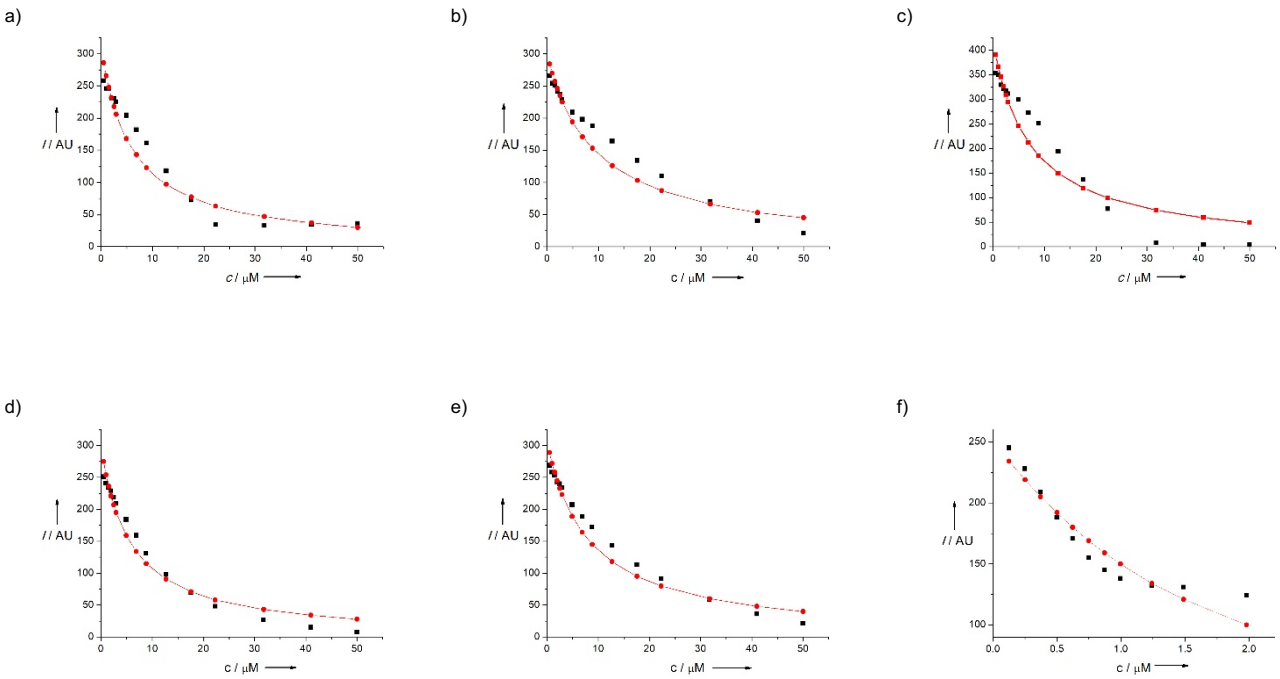



**Figure S33:** Representative plots of the FID binding affinity determination of A) *trans-F*$_4$Azo-*(PyDp)*$_2$ to *dsDNA*$_{hAT,}$ B) *cis-F*$_4$Azo-*(PyDp)*$_2$ to *dsDNA*$_{hAT,}$ C) *trans-F*$_4$Azo-*(PyDp)*$_2$ to *dsDNA*$_{hGC,}$ D) *trans-F*$_4$Azo-*(PyDp)(PyOMe)* to *dsDNA*$_{hAT,}$ E) *cis-F*$_4$Azo-*(PyDp)(PyOMe)* to *dsDNA*$_{hAT}$ and F) netropsin to *dsDNA*$_{hAT,}$ at the wavelength of the maximum of 530 nm. Shown are in black the intensities experimentally determined at different concentrations of DNA and in red the corresponding fit performed by HypSpec.

**Table S6:** Determined mean $K_D$-values and standard deviations of *F*$_4$*Azo-(PyDp)*$_2$ (***2***) *and F*$_4$*Azo-(PyDp)(PyOMe)* (***3***) *as their trans- and cis-isomer and netropsin to dsDNA*$_{hAT}$. *Binding affinities were calculated using the program HypSpec.*

| | *F*$_4$Azo-(PyDp)(PyOMe) (3) | | *F*$_4$Azo-(PyDp)$_2$ (2) | | netropsin |
|---|---|---|---|---|---|
| | *trans* | *cis* | *trans* | *cis* | |
| Mean $K_D$ value | 53.6 ± 2.8 nM | 81.5 ± 6.6 nM | 59.9 ± 1.2 nM | 108.3 ± 14.9 nM | 9.9 ± 0.6 nM |

**Table S7:** Determined mean $K_D$-values and standard deviations of *F*$_4$*Azo-(PyDp)*$_2$ (***2***) *and F*$_4$*Azo-(PyDp)(PyOMe)* (***3***) *in their trans- and cis-conformation to dsDNA*$_{hGC}$. *n.c. stands for non-calculated.*

| | *F*$_4$Azo-(PyDp)$_2$ trans | netropsin |
|---|---|---|
| Mean $K_D$-value | 13.3 ± 0.6 nM | n.c. |

## Control Experiments with $F_4$Azo-$(PyDp)_2$ (2)

To ensure the stability of the *trans*- and the *cis*-state of $F_4$Azo-$(PyDp)_2$ (**2**) during the measurements, we further performed several control experiments.

To show the stability of the isomers of the compound $F_4$Azo-$(PyDp)_2$ (**2**) during the FID measurement, a solution of 50 µM of **2** in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl and DMSO (95:5) (total volume 500 µL) was irradiated in a cuvette for 10 s at 405 nm or 2 min at 520 nm to achieve the maximum *cis/trans* or *trans/cis* conversion. All solutions were kept in the dark and the measurements performed in the absence of light. The cuvette was placed into the fluorimeter and the temperature adjusted to 20 °C. One sample was taken and 30 µL injected into the HPLC. The cuvette was placed back in the fluorimeter, which is used in the FID measurements. 5 fluorescence measurement points were performed and a new 30 µL sample was taken to inject into the HPLC. The same procedure was repeated after 10 and 15 measurement points. Figure S34 illustrates the chromatograms, which show the stability of the *trans*- and the *cis*-state during the FID measurement.

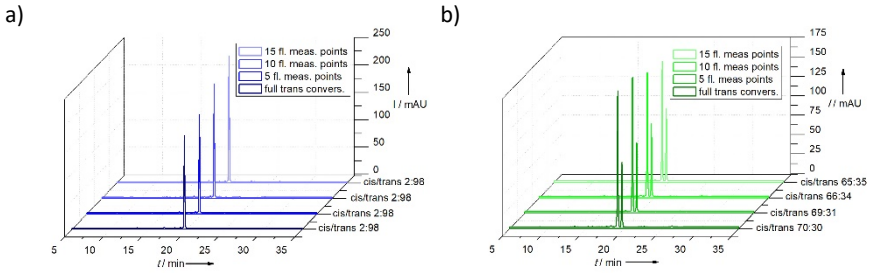



**Figure S34**: *RP-HPLC chromatogram of $F_4$Azo-$(PyDp)_2$ (**2**) show the stability after irradiation at A) 405 nm for 10 s and B) 520 nm for 2 min and their corresponding trans/cis-ratio during the FID measurements. Samples were taken before the measurement and after 5, 10 and 15 measurement points. Represented data are a representative one of three measurements from independent experiments*

To analyze the stability of both isomers of $F_4$Azo-$(PyDp)_2$ (**2**) during the gel mobility shift assay, a solution of 50 µM of $F_4$Azo-$(PyDp)_2$ (**2**) 1X SDB (10 mM Tris, 1 mM EDTA pH 8.0, 50 mM NaCl) and DMSO (95:5) was irradiated for 10 s at 405 nm or 2 min at 520 nm to achieve the maximum *cis/trans* or *trans/cis* conversion. Afterwards it was stored in total darkness at r.t. and RP-HPLC chromatograms, under the same conditions as before were recorded after different time periods (figure S35).

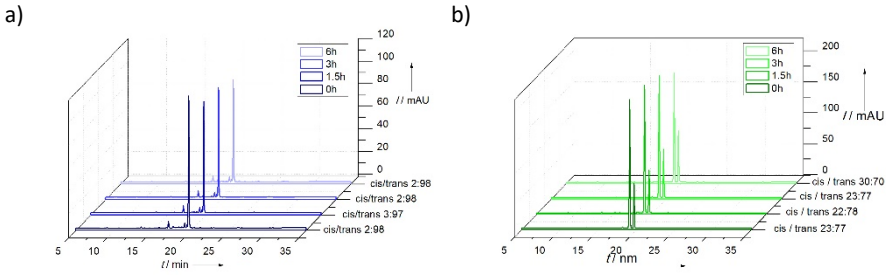



**Figure S35**: *RP-HPLC chromatogram of $F_4$Azo-$(PyDp)_2$ (**2**) under mobility shift assay conditions show the dark stability after irradiation at A) 405 nm for 10 s and B) 520 nm for 2 min and their corresponding trans/cis-ratio after several time points. Represented data are a representative one of three measurements from independent experiments*

To analyze the thermal stability of $F_4$Azo-(PyDp)$_2$ (**2**), a solution of 50 µM of $F_4$Azo-(PyDp)$_2$ (**2**) in 10 mM Tris pH 7.4, 10 mM NaCl and DMSO (95:5) was irradiated for 30 s at 405 nm to achieve the maximum *cis/trans* conversion. Afterwards the temperature was increased with 0.5°C/min and RP-HPLC chromatograms, under the same conditions as before, were recorded after different temperature points (figure S36).

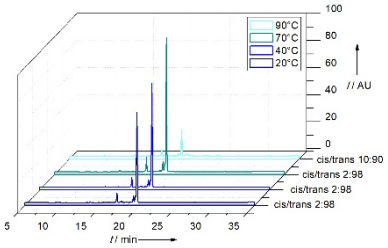



**Figure S36**: *RP-HPLC chromatogram of $F_4$Azo-(PyDp)$_2$ (**2**) after several temperature points. Represented data are a representative one of three measurements from independent experiments*

To check how much the DMSO influences the FID measurements, a FID measurement, like described above was performed, but adding DMSO instead of the DMSO stocks of the compounds to the solution of 6 µM of TO and a 1 µM of *ds*DNA$_{hAT}$ in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl (total volume 1 mL). The maximum at 530 nm was plotted against the added amount of compound, which equals the added DMSO amount. In the plot of the fluorescence intensity against the added compound concentration, the mean value and its standard deviation was calculated out of three independent measurements. The plots are shown in figure S37.

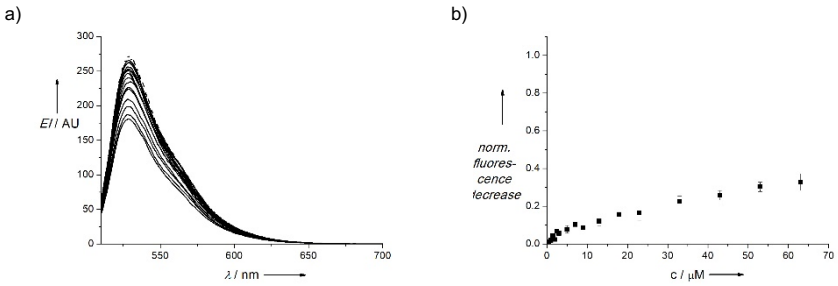



**Figure S37:** A) *dashed line: mean fluorescence emission spectra of a 1 µM TO and 6 µM dsDNA$_{hAT}$ solution in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl. Solid lines: mean fluorescence emission spectra of solutions of a 6 µM TO and 1 µM dsDNA$_{hAT}$ solution in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl and increasing amounts of DMSO. B) Fluorescence intensity maximum at 530 nm plotted against the concentration of added compound, which equal the added amount of DMSO. Mean values with their standard deviation are shown. Represented data and standard deviations are calculated from three independent experiments*

To rule out quenching effects, the influence of the added $F_4$Azo-(COOH)$_2$ (**5**) was checked, by following the same procedure like described for the FID titrations. The plots are shown in figure S38.

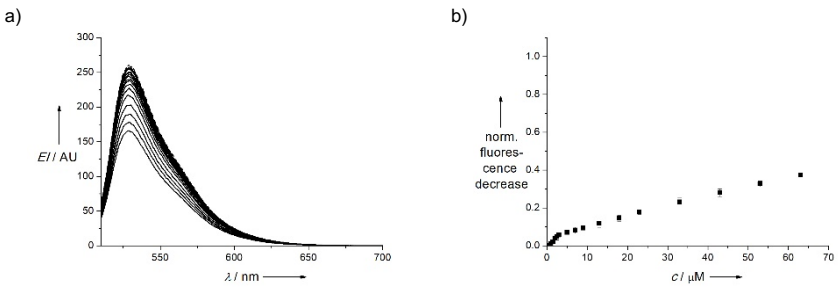



**Figure S38: :** A) *dashed line: mean fluorescence emission spectra of a 1 µM TO and 6 µM dsDNA$_{hAT}$ solution in 20 mM NaH$_2$PO$_4$ pH 7.4, 100 mM NaCl. Solid lines: mean fluorescence emission spectra of solutions of a 6 µM TO and 1 µM dsDNA$_{hAT}$ solution in 20 mM NaH$_2$PO$_4$ pH 7.4, 100 mM NaCl and increasing amounts of F$_4$Azo-(COOH)$_2$ (**5**). B) Fluorescence intensity maximum at 530 nm plotted against the concentration of added compound. Mean values with their standard deviation are shown. Represented data and standard deviations are calculated from three independent experiments.*

## CD Experiments

For the characterization of the binding of compound *F$_4$*Azo-(PyDp)$_2$ (**2**) via circular dichroism, a solution of 50 µM dsDNA$_{CT}$ in 20 mM NaH$_2$PO$_4$ pH 7.4, 100 mM NaCl was prepared in the corresponding cuvette and measured. Solutions of *F$_4$*Azo-(PyDp)$_2$ were prepared as a DMSO stock with a concentration of either 250 µM or 1 mM. These stock solutions were irradiated for 10 s at 405 nm to measure the *cis*-derivative or 2 min at 520 nm to measure the *trans*-derivative. Solutions were kept in the dark and the measurement performed in the absence of light. Small aliquots of the compound stocks were stepwise added and the fluorescence spectra recorded after an incubation time of 3 min. The corresponding spectra are buffer subtracted and show the mean out of two measurements. Figure S39 shows the CD spectra of the *calf thymus* DNA after buffer subtraction. Represented data are mean values calculated from two independent experiments.

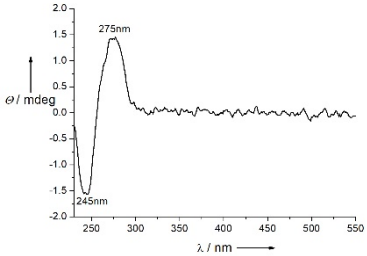



**Figure S39:** *Buffer subtracted CD spectra of a 50 µM solution dsDNA$_{CT}$ in 20 mM NaH$_2$PO$_4$ pH 7.4, 100 mM NaCl. Represented data are mean values calculated from two independent experiments.*

We further recorded the CD spectra of a solution of 5 µM dsDNA$_{AT}$ and dsDNA$_{GC}$ in 20 mM NaH$_2$PO$_4$ pH 7.4, 100 mM NaCl. The procedure were equal the described above. The corresponding spectra are buffer subtracted. Represented data are mean values calculated from two independent experiments (figure S40).

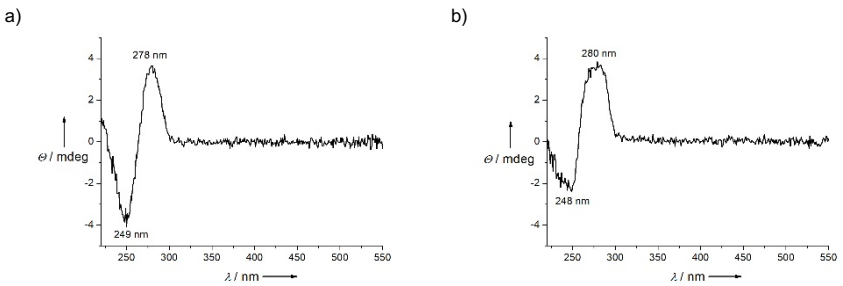



**Figure S40: :** *Buffer subtracted CD spectra of a 5 µM solution of A) dsDNA$_{AT}$ and B) dsDNA$_{GC}$ in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl. Represented data are mean values calculated from two independent experiments*

We further titrated the DNA with increasing amount of spermine. The procedure and concentrations were equal the described above. The spectra were buffer subtracted (figure S41).

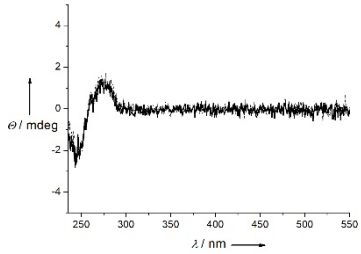



***Figure S41:*** *Buffer subtracted CD spectra of the titrations of a 50 µM solution dsDNA$_{CT}$ in 20 mM $NaH_2PO_4$ pH 7.4, 100 mM NaCl with increasing amounts of spermine where the added equivalents are 0, 0.16, 0.5, 1.1 and 2.8 per mol of dsDNA$_{CT}$.* Represented data are mean values calculated from two independent experiments.

## DLS Experiments

In order to study the effect of *trans*-$F_4$(PyDp)$_2$ (*trans*-**2**) and netropsin on the hydrodynamic diameter of the nucleosome and the nucleosomal DNA we performed DLS measurements. 100 µL of a 2.00 µM nucleosome stock or nucleosomal DNA in 10 mM Tris 1 mM EDTA pH 8.0 and 50 mM NaCl was prepared in the corresponding cuvette and measured. Stocks of *trans*-$F_4$(PyDp)$_2$ (*trans*-**2**, 1.41 mM, 2.94 mM and 5.88 mM) and netropsin (5.88 mM and 11.76 mM) in DMSO were prepared. For every addition, stock volumes of 1.25 µL were stepwise added and the spectra recorded after an incubation time of 3 min. Represented data are a representative one of two measurements from independent experiments. Figure S42 shows the DLS signals of the nucleosome and the free nucleosomal DNA after incubation with different concentrations of netropsin.

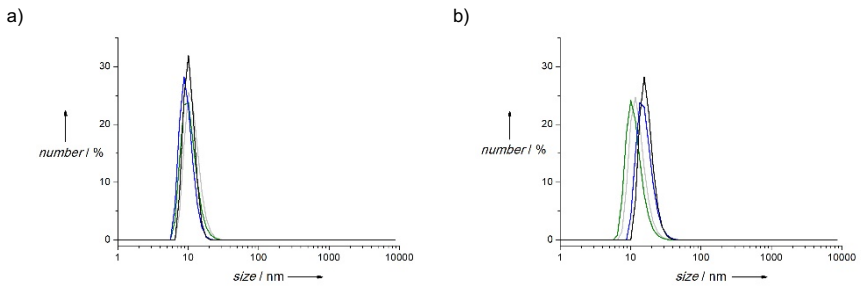



***Figure S42:*** DLS signals of a solution of a) 2 µM nucleosome and b) 2 µM nucleosomal DNA in 10 mM Tris 1 mM EDTA pH 8.0 and 50 mM NaCl with increasing amounts of netropsin, where the equivalents in bp are 0 (grey), 0.25 (green), 0.5 (blue) and 1.0 (black) eq. Represented data are a representative one of two measurements from independent experiments.

For the precipitation by $MgCl_2$, the nucleosome stock was prepared in 3.5 mM Tris 0.35 mM EDTA pH 8.0. For every addition, $MgCl_2$ stock volumes (62.5 mM and 125 mM) in MilliQ water of 1.25 µL were stepwise added and the spectra recorded after an incubation time of 3 min.

To find out at which $MgCl_2$ concentration the nucleosome precipitates, the solutions were analyzed before spectroscopically on a NanoDrop 2000 spectrometer (Thermo Scientific) by measuring the absorbance at 260 nm. Different amounts of $MgCl_2$ were added, the samples incubated for 20 min at r.t. and centrifuged for 20 min at 14000 rpm at r.t. The absorbance at 260 nm of the supernatant was determined. Figure S43 shows the plot of the absorbance against the $MgCl_2$ concentration.

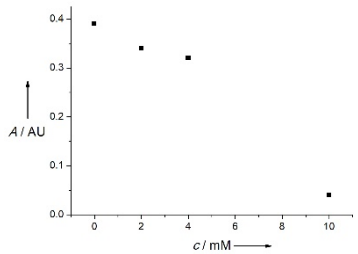



***Figure S43:*** Determined absorbance at 260 nm of the nucleosome in 3.5 mM Tris 0.35 mM EDTA pH 8.0 after addition of different amounts of $MgCl_2$.

## Nucleosome Assays

For the nucleosome assays nucleosomal DNA was produced by polymerase chain reaction (PCR) or in a large scale by alkaline lysis and the histone octamers extracted from chicken blood. Both were assembled to nucleosomes using salt gradient dialysis. To study the effect of our molecules to the nucleosome, Mobility Shift Assays were performed.

### *Nucleosomal DNA*

The nucleosomal DNA ($dsDNA_{601}$) was obtained in two different ways either in a small scale by PCR, which was used for the assembly of nucleosomes and the EMSA studies or in a big scale by a large scale plasmid preparation and further EcoRV-digest for the assembly of higher concentrated nucleosomes, which was used for the DLS experiments.

***Small scale:***

The Plasmid pGEM-3z/601 (Addgene plasmid # 26656, deposited by Jonathan Widom) was used as nucleosomal DNA template. This plasmid was replicated in DH5 alpha cells to facilitate long-term storage and sequencing, Thus, this plasmid was diluted from an initial concentration of 1.1 µg/µL to a final one of 0.1 µg/µL. 0.5 µL of this solution (containing 50 ng of the plasmid) was added to 50 µL of competent cells (*E. coli* DH5 alpha). The cells were then transferred into a 2 mm-gap cuvette for electroporation at 2.5 kV with a 5.8 ms pulse. 1 mL of LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) was added to the cells immediately, and the cells were recovered by incubation at 37°C and 800 rpm for 1 h. Afterwards, cells were plated onto ampicillin containing agar, and grown overnight at 37°C. A streak of bacteria was transferred into 5 mL of LB medium containing ampicillin, and grown for 24 h at 37°C and 200 rpm. For long-term storage, 300 µL glycerol (80%) was added to 700 µL of the bacterial culture, mixed and shock-frozen in liquid nitrogen, after which the glycerol stock was stored at -80°C. The plasmid for amplification was performed from 4 mL of bacterial culture with a GenElute HP Plasmid Miniprep Kit (Sigma Aldrich) by following the manufacturer's instructions. The plasmid was eluted in 50 µL millipore water.

Afterwards, the plasmid was amplified by polymerase chain reaction (PCR). The reagents described in table S8 were mixed and the PCR run with the temperature program indicated in table S9. After finishing the PCR cycles, the products were purified using a GenElute PCR CleanUp Kit (Sigma Aldrich) and eluted with water. The DNA concentration was determined by NanoDrop. The yield from a 1 mL reaction volume was 24.2 µg of DNA.

**Table S8:** List of reagents used for the polymerase chain reaction of Widom 601 DNA.

| reagent | amount |
|---|---|
| HF buffer | 1X |
| DMSO | 3% |
| dNTPs | 200 µM each |
| forward primer | 0.5 µM |
| reverse primer | 0.5 µM |
| plasmid pGEM-3z/601 | 1 ng/µL |
| phusion polymerase | 1 U / 50 µL |

**Table S9:** Temperature program for the polymerase chain reaction of Widom 601 DNA.

| | |
|---|---|
| initial denaturation | 98 °C / 30 s |

| | |
|---|---|
| denaturation | 98 °C / 8 s |
| annealing | 60 °C / 20 s |
| elongation | 72 °C / 10 s |
| number of cycles | 30 |
| final elongation | 72 °C / 4 min |
| | 4 °C / hold |

**Table S10:** primer sequences used in the polymerase chain reaction of Widom 601 DNA.

| primer | sequence |
|---|---|
| forward primer | CCTGGAGAATCCCGGTGC |
| reverse primer | CAGGATGTATATATCTGACACGTGCC |

***Large scale:***

The Plasmid pUC-19/16x601 (deposited by Ji-Joon Song) was used as nucleosomal DNA template. This plasmid was replicated in DH5 alpha cells and grown on agar plates as described above. A streak of bacteria was transferred into 6 mL of LB medium containing ampicillin, and grown for o/n at 37°C and 200 rpm. 1 mL each was transferred into 1 L of LB medium containing ampicillin, and grown for o/n at 37°C and 200 rpm. The alkaline lysis plasmid preparation was performed following a protocol of Luger *et al*.[xviii] For the purification of the insert, 5.44 mg plasmid at a concentration of 1.0 µg/mL in 10 mM Tris HCl pH 8.5 10 mM $MgCl_2$ 100 mM KCl and 0.1 mg/mL BSA was treated with 700 U EcoRV (Thermo Fisher) at 37°C o/n and additional 70 U EcoRV for 7 h at 37°C. The purification was performed following the same protocol as described above. As a final purification step, gel filtration was included. SEC was performed on an Äkta FPLC system (Amersham Biosciences) equipped with pump P-920, mixer M-925, sample injection valve INV-907, fraction collector Frac-950, monitor UPC-900 and remote connector CU-950, controlled via Unicorn V5.0 software, using a Superdex 200 10/300 GL-column by GE Healthcare with a flow of 0.5 mL/min in 10 mM Tris 1 mM EDTA pH 8.0 and 150 mM NaCl. Detection was executed at a wavelength of 254 nm. The final product fractions were concentrated using a 10000 MWCO Amicon Ultra-15 Centrifugal Filter (Merck).

## *Native Polyacrylamide Gel Electrophoresis*

To analyze molecular weights of DNA and nucleosomes and to perform the EMSA, native polyacrylamide gel electrophoresis (native PAGE) was performed. Polyacrylamide gels were prepared as 5% gels in 0.5X TBE (10 mM Tris, 45 mM borate, 1 mM EDTA). Therefore, 1.875 µL 40% acrylamide solution (19 : 1, acrylamide : bisacrylamide) was diluted in 0.5X TBE to a total volume of 15 mL. The reaction was started by addition of 150 µL of an APS solution (10% in water) and 7.5 µL TEMED. The mixture was immediately poured into commercially available gel cassettes (glas, 1.00 mm, Biorad) placed in a Tetra Cell Casting Stand (Biorad), where wells were created by comb insertion. After complete polymerization (approximately 40 min), the comb was removed, the glass plates and wells washed with MilliQ water and the cassette placed into an electrophoresis chamber filled with 0.5X TBE. The wells were flushed with the running buffer by pipette. After adding 0.1 volumes glycerol to the samples, 20 µL containing 0.06 µM DNA or 0.27µM nucleosomes were loaded into the wells. The gel was run at 90 V for 40 min in a Ready Gel Cell (Biorad) using a Power Pac 300 (Biorad). The gel was poststained for 15 min in an ethidium bromide solution (50 µL of 0.1% solution in 100 mL 0.5X TBE buffer) and visualized under UV-light using a ChemiDoc MP Imaging System (BioRad).

## *Histone Octamer Extraction*

Chicken erythrocyte histone octamers were extracted from chicken blood, following a procedure of Smale *et al*.[xix] Divergent to the protocol, the lysis of the chicken erythrocyte nucleis and the final removal of the DNA was modified. The lysis of the chicken erythrocyte nuclei was performed by sonication 5 times 30 s at 30% with a Sonopuls Ultrasonic Homogenizer HD 2200 (Bandelin) with 2 min rest in between on ice until all clumps were converted into a homogeneous solution. To remove remaining DNA from the chicken histones, purification by hydroxyapatite chromatography did not yield in sufficiently pure histone octamers, but remaining DNA. For further purification, the solution was concentrated to 3.5 mg/mL and treated 3 times with 5 mL HT Gel (Biorad). Before use, the HT Gel was washed with wash buffer and then added to the histone solution. After incubation for 3 h at 4°C, the HT Gel was centrifuged and the supernatant collected. Purity was checked by 6% stacking and 15% running SDS-PAGE. Staining was performed with coomassie. To verify the absence of DNA, a gel performed in the same way was stained with EtBr. Figure S44 shows the SDS-PAGE of the final histone octamers.

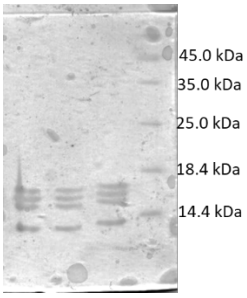



**Figure S44:** SDS-PAGE of the purified histone octamers.

## *Reconstitution of Nucleosome Core Particles*

Nucleosome core particles were reconstituted by salt gradient dialysis.[xix] A test assembly to experimentally verify the amount of chicken histones necessary. to nucleosome reconstitution was performed in a final concentration of 1 µg DNA per 30 µL reaction

volume. Such DNA amount was mixed in 1X SDB (10 mM Tris, 1 mM EDTA pH 8.0 and 2 M NaCl) with chicken histone octamers at different concentrations: volumes between 9.75 µL and 11.25 µL in 0.25 µL steps of a histone octamer stock of 47 µg/mL were added. These solutions were given in dialysis cells (Nadir-dialysis tubing, cellulosehydrate, molecular weight cut-off: 10 – 20 kDa, Carl Roth) and dialyzed in 1X SDB for 1 h. The NaCl concentration was step-wise reduced (2 M → 1 M → 500 mM → 50 mM; dialysis time each 1 h) while keeping the other buffer components constant. The final dialysis step was performed for 16 h. The nucleosomes were taken out of the dialysis chamber, centrifuged at 10000 rpm for 2 min and the supernatant analyzed by EMSA. Figure S45 shows the native PAGE gel electrophoresis mobility shift assay (EMSA) analyzed by EtBr-staining of the test assembly, were conditions 3 were found to be the ideal conditions.

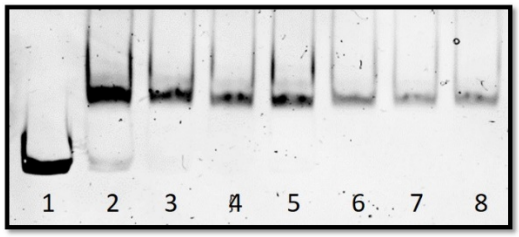



**Figure S45**: *EMSA results showing the test assembly of the nucleosome. Experiments were carried out with 0.06 µM of free DNA or 0.27 µM of nucleosome. All experiments were carried out in the following buffer solution: 10 mM Tris, 1 mM EDTA pH 8.0 and 50 mM NaCl. 1 marks the free DNA, 2-8 mark the test assembly conditions of 1 µg DNA mixed with 9.25, 9.50, 9.75, 10.00, 10.25, 10.50, 10.75 µL of a histone octamer stock of 47 µg/mL.*

For the large scale nucleosome assembly, the optimal conditions from above were used to assemble enough nucleosomes. The assembly was performed under the same concentrations and conditions, but in a reaction volume of 600 µL. Purity of the reconstituted nucleosome core particles was checked by EMSA.

For higher concentrated nucleosomes, which were necessary for DLS, nucleosomes were reconstituted at a concentration of 10 µg DNA per 30 µL reaction volume. The procedure matches the above described one.

## *Gel Electrophoresis Mobility Shift Assay (EMSA)*

To verify nucleosome stability after incubation with $F_4$Azo-$(PyDp)_2$ (**2**) and $F_4$Azo-(PyDp)(PyOMe) (**3**) EMSA was performed. To 20 µL of a 0.3 µM solution of nucleosomes (40 µM bp after DMSO stock addition) in 10 mM Tris pH 8.0, 1 mM EDTA and 50mM NaCl, stock solutions of the compound (**2**) or (**3**) were added in the dark. Stock solutions were prepared in DMSO and irradiated for 10 s at 405 nm or 2 min at 520 nm to obtain the *trans*- or *cis*-isomer. Solutions were kept in the dark and the measurement performed in the absence of daylight. Dilution rows were prepared and 2 µL of these stocks added to the nucleosomes. The DMSO stocks had an end concentration of 1.76, 0.88, 0.44, 0.22, 0.11, 0.055, 0.028, 0.014 mM (this equals 4, 2, 1, 0.5, 0.25, 0.13, 0.06, 0.03 eq in bp). As controls, 55 nM free DNA (8 µM bp) and 0.27 µM nucleosome (40 µM bp) in 10 mM Tris, 1 mM EDTA pH 8.0 and 50mM NaCl and DMSO (90:10) were prepared. The solutions were incubated for 3 h at r.t., 2 µL glycerol added and the samples loaded on the native PAGE gel, run and stained with EtBr.

We further incubated the nucleosome with netropsin under the same conditions and same concentrations but without irradiation as well as using higher concentrations of netropsin with the DMSO stocks having an end concentration of 14.1, 7.04, 3.52, 1.76, 0.88 mM (this equals 32, 16, 8, 4, 2 eq in bp).

We further performed the nucleosome binding assys with in situ irradiation. For this purpose we chose conditions with a clear difference in nucleosome stability for the *trans*- and *cis*-isomer of $F_4$Azo-$(PyDp)_2$ (**2**). Following the procedure described above we

incubated two reference samples of 40 µM bp nucleosome with 0.5 and 0.25 eq of $F_4$Azo-(PyDp)$_2$ (**2**) in *trans*- and *cis*-conformation for 3 h. Another three nucleosome samples were incubated with 0.5, 0.25 and 0.13 eq of $F_4$Azo-(PyDp)$_2$ (**2**) in *cis*-conformation for 1 h, irradiated these samples for 10 s at 405 nm and incubated for another 2 h.

To validate the influence of the irradiation during the in situ irradiation experiment on the nucleosome binding, we prepared samples of 40 µM bp nucleosome in 20 µL 10 mM Tris, 1 mM EDTA pH 8.0 and 50mM NaCl and added 2 µL of DMSO. The samples were irradiated for 1 s / 5 s / 10 s / 30 s / 1 min / 5 min at 405 nm and the EMSA performed.

## *EMSA Controls of the DLS*

During the measurements of the DLS, aliquots of each concentration steps were taken, to analyze them by EMSA. 2 µL of the 2 µM solution were mixed with 0.5 µL of glycerol and loaded on a 5% native PAGE 0.5X TBE. The gel was performed like described above. Analysis after EtBr revealed the results presented in figure S46.

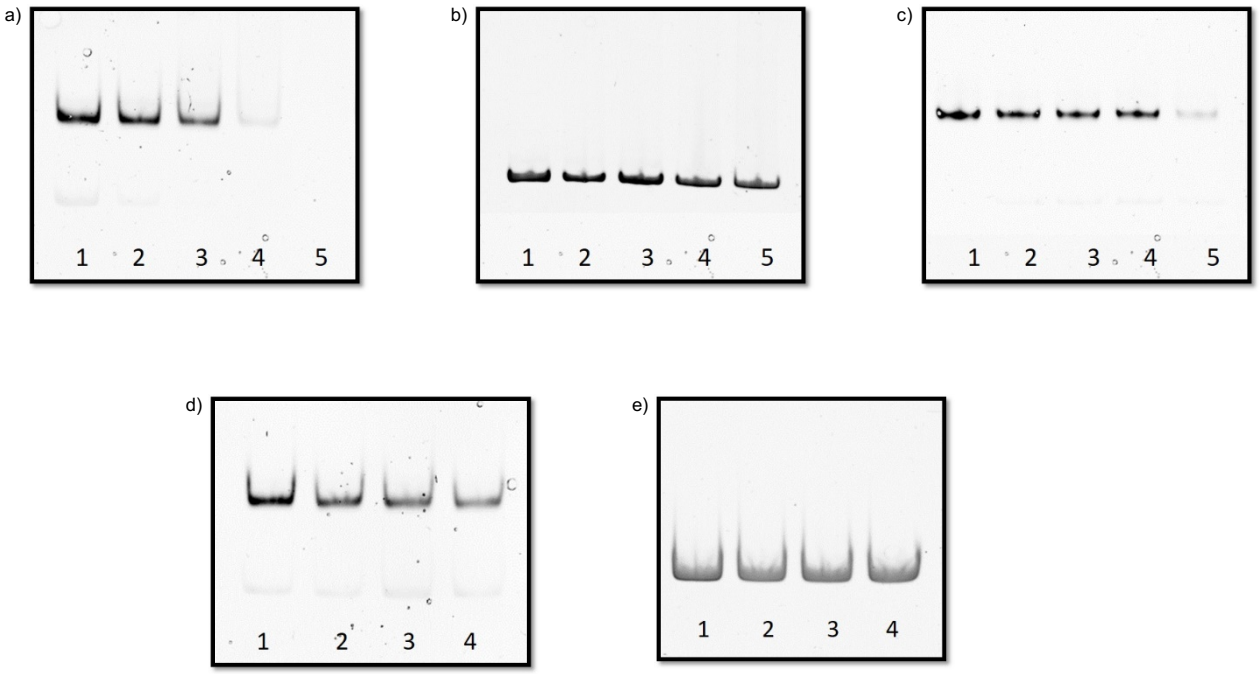



**Figure S46:** *EMSA results showing the NCP after incubation with a) trans-$F_4$(PyDp)$_2$, c) $MgCl_2$ and d) netropsin at different concentrations and the free nucleosomal DNA after incubation with b) trans-$F_4$(PyDp)$_2$ and e) netropsin at different concentrations. Molar equivalents of the compounds per mol bp are in a and b) 0, 0.06, 0.13, 0.25 and 0.50 eq;in d) and e) 0, 0.25, 0.50, 1.0 eq. $MgCl_2$ concentrations in c) are 0, 2.5, 3.75, 5.0, 10.0 mM. 2 µL aliquots of the sample were taken during the DLS measurement, mixed with 0.5 µL glycerol, run on a 5% native PAGE 0.5X TBE and stained with EtBr.*

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