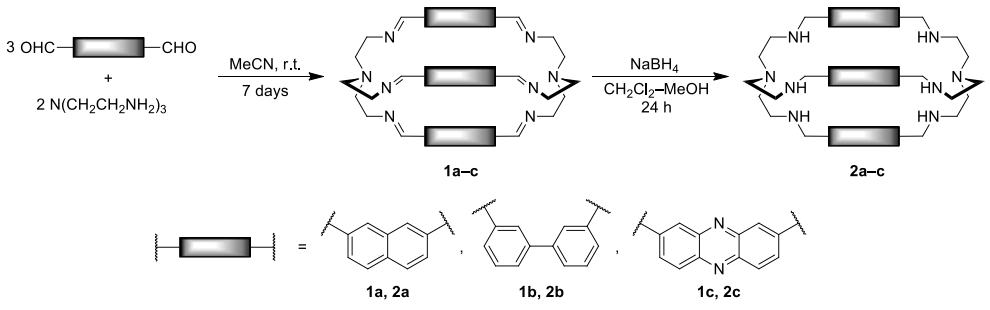
Cationic azacryptands as selective three-way DNA junction binding agents

Jana Novotna,[a,b] Aurelien Laguerre,[a] Anton Granzhan,[c] Marc Pirrotta,[a] Marie-Paule Teulade-Fichou[c] and David Monchaud[a,*]

[a] Institute of Molecular Chemistry, University of Dijon, ICMUB CNRS UMR6302, Dijon, France.
[b] Department of Analytical Chemistry, Institute of Chemical Technology, Prague, Czech Republic.
[c] Institut Curie, Université Paris Sud, CNRS UMR176, Orsay, France.
*E-mail: david.monchaud@u-bourgogne.fr

Part 1. Chemistry.

General remarks. Aromatic dialdehydes were prepared according to the published procedures (*cf*. A. Granzhan, E. Largy, N. Saettel, M.-P. Teulade-Fichou, *Chem. Eur. J.* **2010**, *16*, 878–889). NMR spectra were measured with a Bruker Avance 300 (^{1}H: 300 MHz, ^{13}C: 75 MHz) spectrometer at 25 °C; chemical shifts (δ) are given in ppm. The multiplicities of ^{13}C NMR signals were determined by means of DEPT-135 experiments. Melting points were determined in open-end capillaries with a digital melting point instrument (SMP30, Stuart). Elemental microanalyses of the new compounds were performed by the *Service de Microanalyse*, CNRS-ICSN, Gif-sur-Yvette, France. Mass spectra (ESI in positive-ion mode) were recorded with a Waters ZQ instrument (source voltage: 75 kV).



General procedure for synthesis of macrobicyclic compounds. 1) A solution of dialdehyde (3.00 mmol) in MeCN (for **1a** and **1b**: 150 mL) or 1:1 (v/v) MeCN–CH_2Cl_2 (for **1c**: 300 mL) was added dropwise, under argon, to a vigorously stirred solution of tris(2-aminoethyl)amine (2.00 mmol) in

MeCN (100 mL). The reaction mixture was stirred at room temperature for 7 days and then concentrated, in vacuo, to a half of its initial volume. The precipitated solid was collected, thoroughly washed with MeCN and dried in vacuo, to give the hexaimine intermediate (**1a–c**) which was sufficiently pure and used in the next step without characterization. 2) Sodium borohydride (10 mmol) was added to a stirred suspension of **1a–c** (0.5 mmol) in a mixture of CH_2Cl_2 (30 mL) and MeOH (20 mL). After stirring for 24 h at room temperature, aq. NaOH (1 M, 20 mL) was added, the organic phase was separated, and the aqueous phase was extracted with chlorophorm (3 × 20 mL). The combined organic phases were washed with satd. aq. Na_2CO_3 (20 mL), dried over K_2CO_3, and the solvent was removed in vacuo, to give the crude macrocbicycle **2a–c**. Compounds **2a** and **2b** were isolated and characterized as hydrochloride salts: the crude product was dissolved in 1,4-dioxane (20 mL), treated with excess HCl (4 M in 1,4-dioxane), the volatiles were removed in vacuo, and the residue was recrystallized in water, to give **2a × 6 HCl** or **2b × 7 HCl**, respectively, as analytically pure crystalline solids. Compound **2c** was obtained in analytically pure form after purification by flash chromatography (SiO_2, eluent: CH_2Cl_2–MeOH–aq. NH_4OH, 80:20:1 to 80:20:4), followed by recrystallization from pyridine.

1,4,8,11,14,18,23,27-Octaaza-6,16,25(2,7)-trinaphthalenabicyclo[9.9.9]nonacosaphane hexahydrochloride (**2a × 6 HCl, 2,7-TrisNP × 6 HCl**)**:** yield 31%; colorless needles, m.p. >260 °C; ^{1}H NMR (0.01 M DCl in D_2O): δ = 2.89 (m, 2H), 3.34 (m, 2H), 7.65 (d, J = 8.5 Hz, 1H), 7.78 (s, 1H), 8.01 (d, J = 8.5 Hz, 1H); ^{13}C NMR (0.01 M DCl in D_2O): δ = 46.3 (CH_2), 51.1 (CH_2), 52.3 (CH_2), 128.5 (CH), 129.4 (C_q), 129.5 (CH), 130.9 (CH), 133.0 (C_q), 133.8 (C_q); MS (ESI$^+$): m/z (%) = 375.5 (46) $[M + 2H]^{2+}$, 749.5 (100) $[M + H]^+$, 785.5 (8) $[M + HCl + H]^+$; anal. calc. (%) for $C_{48}H_{60}N_8$ × 6 HCl × 1.5 H_2O (994.8): C, 57.95; H, 6.99; N, 11.26; Cl, 21.38; found: C, 57.88; H, 6.93; N, 11.05; Cl, 21.21.

1,4,9,12,15,20,25,30-Octaaza-6,7,17,18,27,28(1,3)-hexabenzenabicyclo[10.10.10]dotriacontaphane heptahydrochloride (**2b × 7HCl, 3,3′-TrisBP × 7 HCl**)**:** yield 21%; colorless needles, m.p. > 260 °C; ^{1}H NMR (0.01 M DCl in D_2O): δ = 2.83 (t, J = 6 Hz, 2H), 3.16 (t, J = 6 Hz, 2H), 4.19 (s, 2H), 7.41 (d, J = 8 Hz, 1H), 7.49 (dd, J = 8 Hz, 1H), 7.75 (d, J = 8 Hz, 1H), 7.80 (s, 1H); ^{13}C NMR (0.01 M DCl in D_2O): δ = 44.4 (CH_2), 49.7 (CH_2), 51.5 (CH_2), 128.9 (CH), 129.1 (CH), 130.1 (CH), 130.7 (CH), 131.7 (C_q), 141.0 (C_q); MS (ESI$^+$): m/z (%) = 414.5 (100) $[M + 2H]^{2+}$, 827.5 (11) $[M + H]^+$, 863.5 (22) $[M + HCl + H]^+$; anal. calc. (%) for $C_{54}H_{66}N_8$ × 7 HCl (1082.4): C, 59.92; H, 6.80; N, 10.35; Cl, 22.93; found: C, 60.05; H, 6.81; N, 10.31; Cl, 22.99.

1,4,8,11,14,18,23,27-Octaaza-6,16,25(2,8)-triphenazinabicyclo[9.9.9]nonacosaphane (**2c, 1,8-TrisPZ**)**:** yield 26%; brown needles, m.p. 245–247 °C (dec.); ^{1}H NMR ($CDCl_3$): δ = 2.43 (br s, 1H), 2.81 (t, J = 5 Hz, 2H), 3.00 (t, J = 5 Hz, 2H), 3.91 (s, 2H), 7.28 (br s, 2H), 7.51 (s, 1H); ^{13}C NMR ($CDCl_3$): δ = 47.8 (CH_2),

52.9 (CH_2), 54.3 (CH_2), 126.9 (CH), 127.9 (CH), 130.4 (CH), 141.0 (C_q), 141.3 (C_q), 141.7 (C_q); MS (ESI^+): *m/z* (%) = 905.8 (100) $[M + H]^+$; anal. calc. (%) for $C_{54}H_{60}N_{14}$ (905.2): C, 71.65; H, 6.68; N, 21.66; found: C, 71.45; H, 6.86; N, 21.69.

Part 2. Oligonucleotides.

Sequences (from 5' to 3'), extinction coefficient (ε) values expressed in $M^{-1}.cm^{-1}$

Labeled DNA:

fam*-TWJ-*tamra (ε: 435500): *fam*-ACTCTTCTCG-TTTTTT-CGAGAGCGAC-TTTTTT-GTCGCAGAGT-*tamra*

fam*-DS-*tamra (ε: 258900): *fam*-TATAGCTATA-TTTTTT-TATAGCTATA-*tamra*

fam*-GQ-*tamra (**F21T**) (ε: 268300): *fam*-GGGTTAGGGTTAGGGTTAGGG-*tamra*

Unlabeled DNA:

ds26 (ε: 235200): CAATCGGATCGAATTCGATCCGATTG (*self-complementary sequence*)

TG5T (ε: 67900): TGGGGGT

PAGE TWJ-S1 (ε: 131900): CGGAACGGCACTCG

PAGE TWJ-S2 (ε: 135200): CGAGTGCAGCGTGG

PAGE TWJ-S3 (ε: 118700): CCACGCTCGTTCCG

CD TWJ-S1 (ε: 196400): GGTGGCGAGAGCGACGATCC

CD TWJ-S2 (ε: 193800): GGATCGTCGCAGAGTTGACC

CD TWJ-S3 (ε: 173900): GGTCAACTCTTCTCGCCACC

CD-melting TWJ (ε: 382200): ACTCTTCTCG-TTTTTT-CGAGAGCGAC-TTTTTT-GTCGCAGAGT

Preparation of the oligonucleotide stock solutions. The lyophilized strands are firstly diluted in deionized water (18.2 MΩ.cm resistivity) at 500µM for monomolecular and bimolecular DNA constitutive strands and at 1000µM for trimolecular and tetramolecular DNA constitutive strands. The actual concentration of stock solutions is determined by a dilution to 1µM theoretical concentration and a UV-Vis spectra analysis at 260nm (after 5min at 90°C) using the molar extinction coefficient values provided by the manufacturer (*vide supra*).

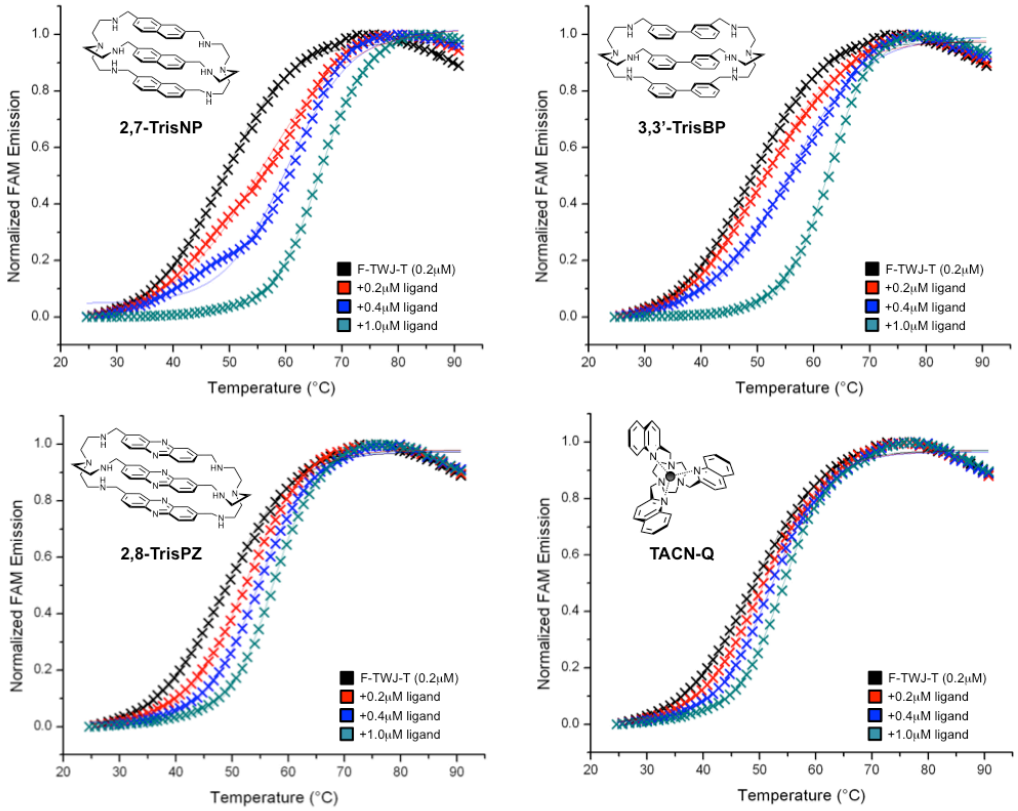
Preparation of the DNA structures. All DNA structures are prepared in a Caco.K buffer, comprised of 10mM lithium cacodylate buffer (pH 7.2) plus 10mM KCl/90mM LiCl. Monomolecular structures are prepared by mixing the constitutive strand (40µL at 500µM) with lithium cacodylate buffer solution (8µL, 100mM, pH 7.2), plus KCl/LiCl solution (8µL, 100mM/900mM) and water (24µL). Bimolecular structures are prepared by mixing each of the two constitutive strands (40µL, 500µM) with lithium cacodylate buffer solution (16µL, 100mM, pH 7.2), plus KCl/LiCl solution (16µL, 100mM/900mM) and water (48µL). Trimolecular structures are prepared by mixing each of the three constitutive strands (20µL, 1000µM) with lithium cacodylate buffer solution (12µL, 100mM, pH 7.2), plus KCl/LiCl solution

(12µL, 100mM/900mM) and water (36µL). Tetramolecular structures are prepared by mixing each of the four constitutive strands (20µL, 1000µM) with lithium cacodylate buffer solution (32µL, 100mM, pH 7.2), plus KCl/LiCl solution (32µL, 100mM/900mM) and water (96µL). The final concentrations are theoretically of 250, 125, 166.7 and 83.3µM for mono-, bi-, tri- and tetra-molecular DNA structures respectively. The actual concentration of each DNA is determined via a dilution to 1µM theoretical concentration (expressed in motif concentration) for monomolecular structure (i.e. 4µL in 996µL water), at 1µM for bimolecular structure (i.e. 8µL in 992µL water), at 0.4µM for trimolecular structure (i.e. 2.4µL in 997.6µL water) and at 0.2µM for tetramolecular structure (i.e. 2.4µL in 997.6µL water), via UV-Vis spectra analysis at 260nm (after 5min at 90°C, baseline correction at 390nm), using the following molar extinction coefficient values: 435500 (*fam*-TWJ-*tamra*), 258900 (*fam*-DS-*tamra*); 268300 (*fam*-GQ-*tamra*); 506400 (ds26); 271600 (TG5T); 385800 (PAGE TWJ); and 382200 $M^{-1}.cm^{-1}$ (CD-melting TWJ).

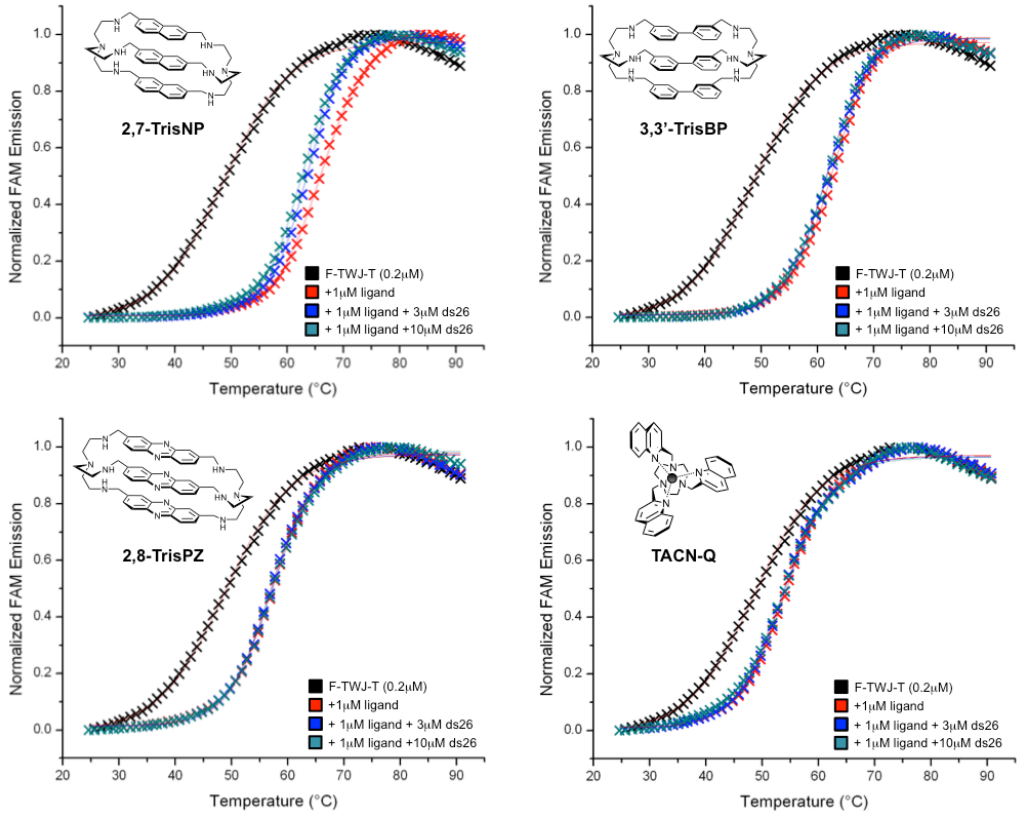
Folding of the higher-order DNA structures. The high-order DNA structures were folded according to two distinct procedures: i- for the intramolecular architectures, the solutions are heated at 90°C for 5min, cooled in ice for 7h, and then stored at least overnight at 4°C; ii- for the folding of all other structures, the solutions are heated at 90°C for 5min, cooled at 80°C for 10min, 60°C for 30min, 40°C for 30min, 25°C for 4h and then stored at least overnight at 4°C.

Part 3. FRET-melting experiments.

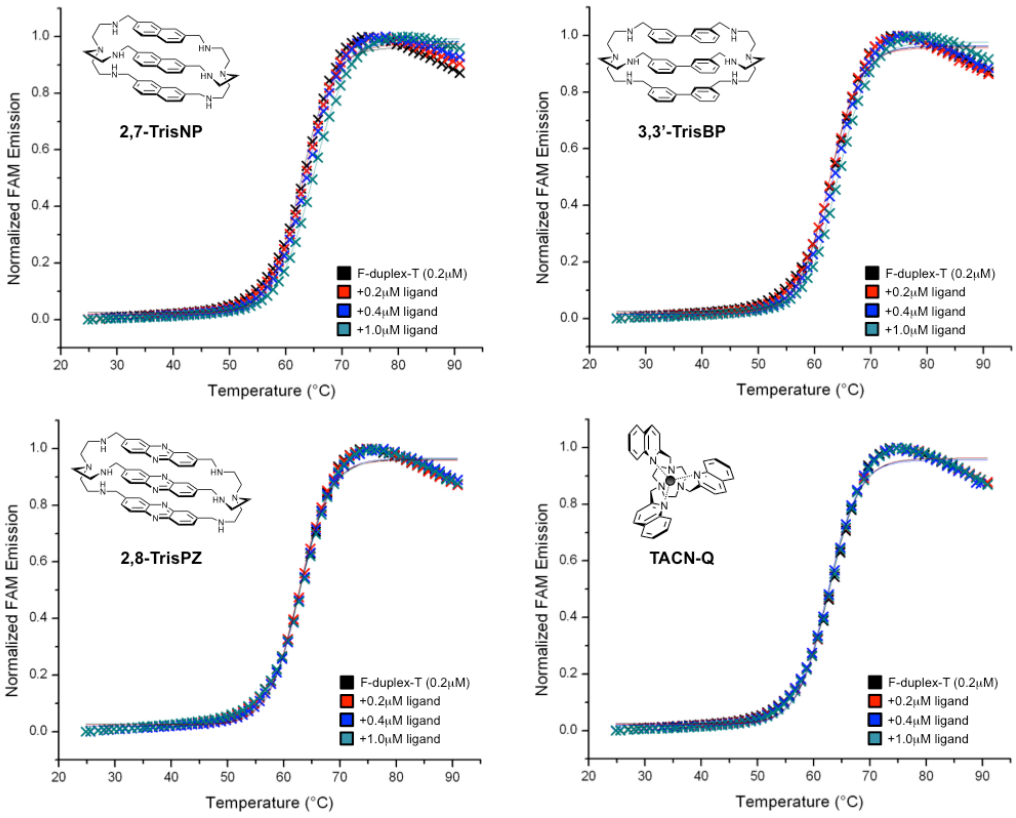
Experiments were performed in a 96-well format using a Mx3005P qPCR machine (Agilent) equipped with a FAM filter (λ_{ex} = 492nm; λ_{em} = 516nm). Dose-response experiments were carried out in 100µL (final volume) of 10mM lithium cacodylate buffer (pH 7.2) + 99mM LiCl/1mM KCl (except for *fam*-DS-*tamra* and *fam*-GQ-*tamra*: 90mM LiCl/10mM KCl), with 0.2µM of labeled DNA and increasing amounts (0 to 5equiv.) of cryptands/TACN-Q (0 to 1µM). Competitive experiments were carried out in 100µL (final volume) of 10mM lithium cacodylate buffer (pH 7.2) + 99mM LiCl/1mM KCl, with labeled DNA (0.2µM), cryptands/TACN-Q (1.0µM) and increasing amounts (0, 15 and 50equiv.) of unlabeled competitors (ds26 or TG5T). After a first equilibration step (25°C, 30s), a stepwise increase of 1°C every 30s for 65 cycles to reach 90°C was performed, and measurements were made after each cycle. Final data were analyzed with Excel (Microsoft Corp.) and OriginPro®8 (OriginLab Corp.). The emission of *fam* was normalized (0 to 1), and $T_{1/2}$ was defined as the temperature for which the normalized emission is 0.5; $\Delta T_{1/2}$ values are means of 2 to 4 experiments.



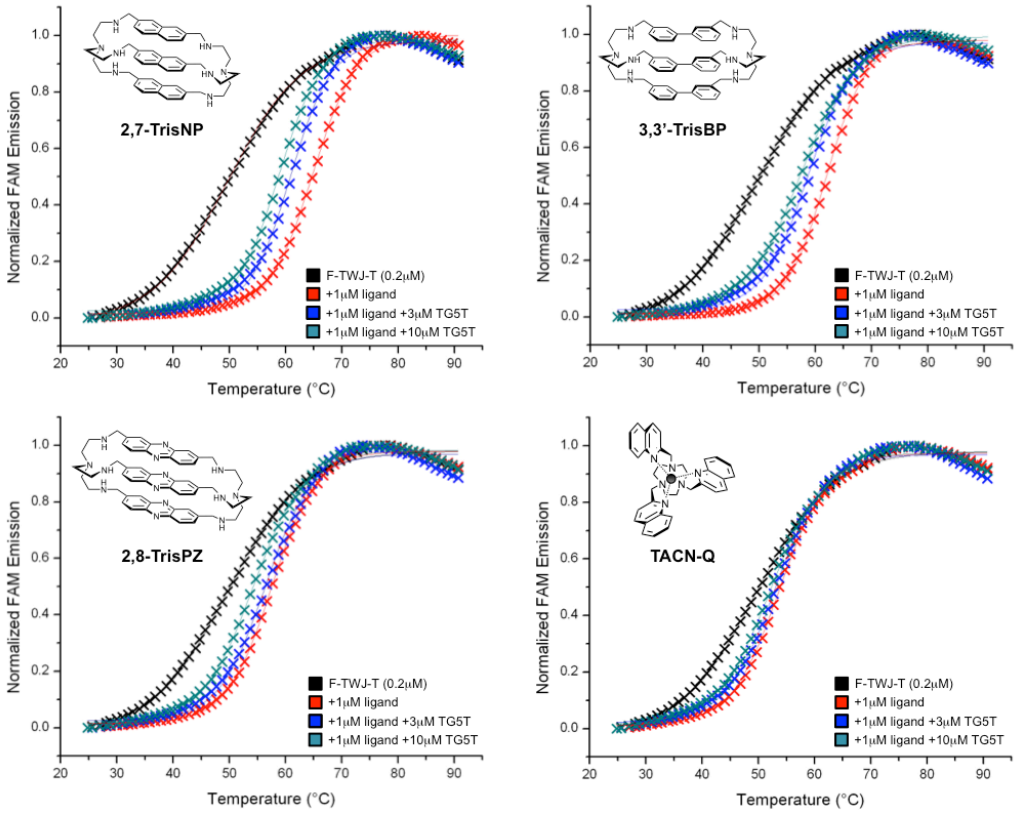
FRET-melting results of experiments carried out with *fam*-TWJ-*tamra* and increasing amounts of ligands



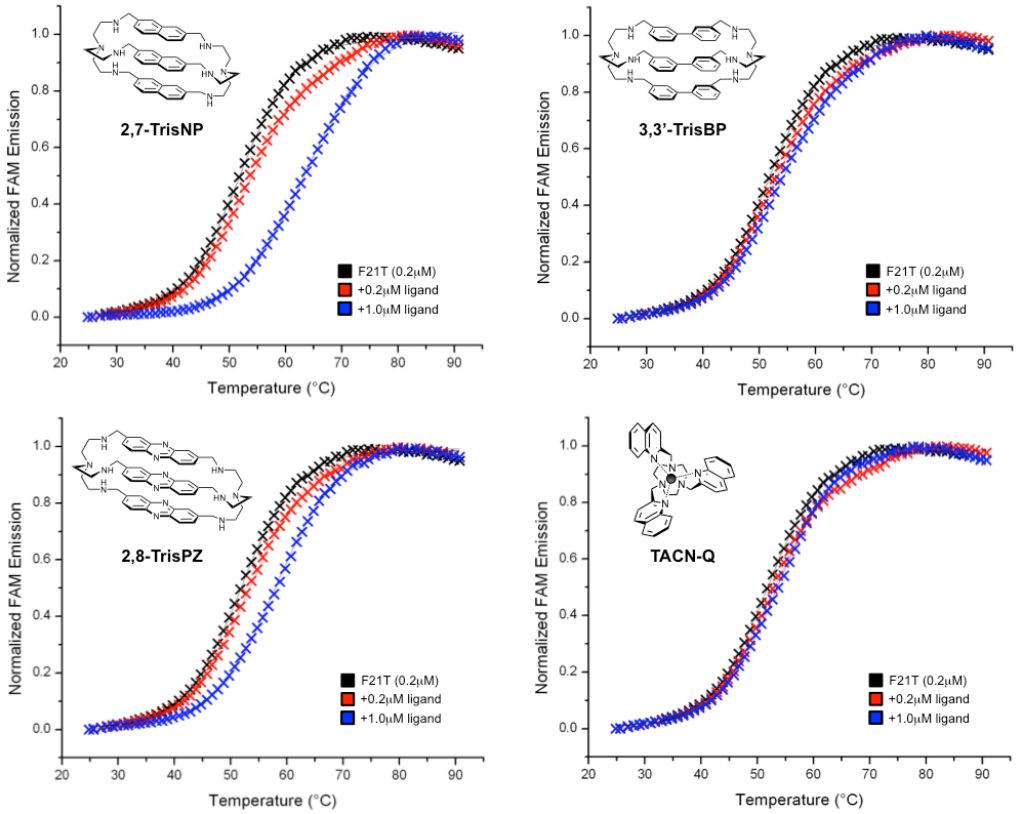
FRET-melting results of experiments carried out with *fam*-TWJ-*tamra*, 5 equiv. of ligands and increasing amounts of a duplex-DNA competitor (ds26)



FRET-melting results of experiments carried out with *fam*-DS-*tamra* and increasing amounts of ligands



FRET-melting results of experiments carried out with *fam*-TWJ-*tamra*, 5 equiv. of ligands and increasing amounts of a quadruplex-DNA competitor (TG5T)



FRET-melting results of experiments carried out with *fam*-GQ-*tamra* (F21T) and increasing amounts of ligands

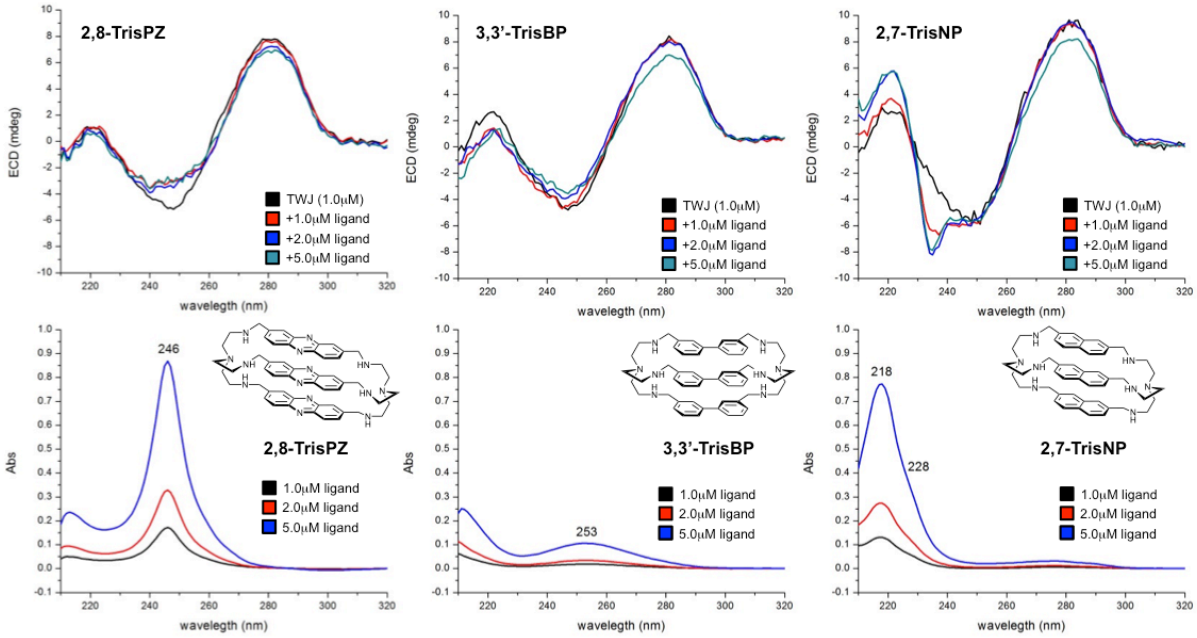
Part 4. Gel electrophoresis.

Nondenaturing polyacrylamide gel electrophoresis was carried out with 20% polyacrylamide-bisacrylamide (19:1) gel. Samples were prepared in 15µL (final volume) of 1xTB buffer + 10mM MgCl, with DNA (5µM) and cryptands, TACN-Q, $AuTMX_2$, ^{PNA}DOTASQ or $TMPyP_4$ (1equiv.). Alternatively, samples were prepared in 15µL (volume) of 1xTB buffer + 10mM MgCl with DNA (5µM) and increasing amounts of either 3,3'-TrisBP or 2,8-TrisPZ (0 to 10equiv.). Loading samples were prepared mixing 5µL of the previously prepared DNA samples (±ligands) and 10µL of sucrose (15% in H_2O; w/v), thus corresponding to a final DNA concentration of 5µM; 10µL of this mixture were thus loaded on the gel. The electrophoretic migration were performed in 1×TB (tris-borate buffer), pH 8.3, for 2.5hr at 4°C (180V). After the migration, gels were analyzed after a post-staining step (SYBR® Safe solution, 1:10000, 10mn, 25°C under gentle agitation) with a UVP MultiDoc-It® imaging system (λ_{ex} = 254nm).

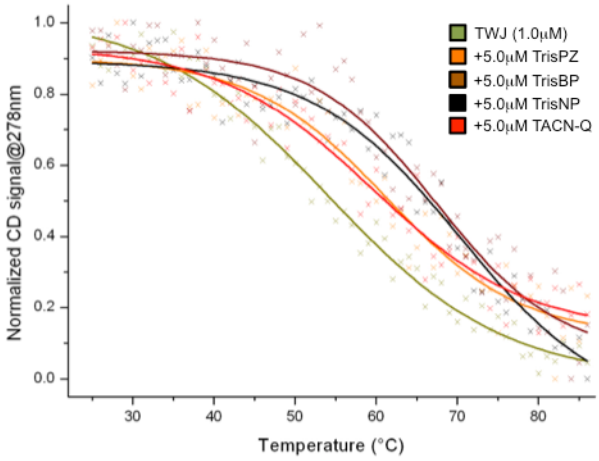
Part 5. Circular dichroism & UV spectroscopies.

CD titrations. CD and UV-Vis spectra were recorded on a JASCO J-815 spectropolarimeter and a JASCO V630Bio spectrophotometer, respectively, in a 10mm path-length quartz semi-micro cuvette (Starna). CD spectra of TWJ (1µM) were recorded over a range of 210-320nm (bandwidth = 1nm, 1nm data pitch, 1s response, scan speed = 100nm.mn^{-1}, averaged over 5 scans, zeroed at 320nm) without

and with cryptands/TACN-Q (1, 2 and 5µM in H_2O) in 0.5mL (final volume) of 10mM lithium cacodylate buffer (pH 7.2). UV spectra were recorded as a function of the ligands concentration (0 to 5µM), in 10mM lithium cacodylate buffer (pH 7.2)



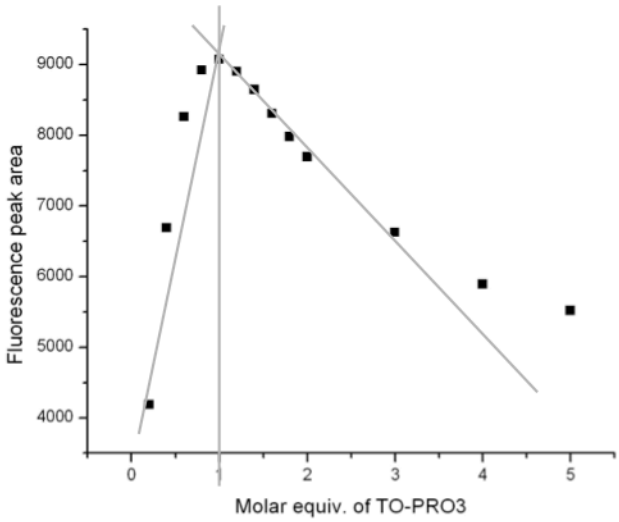
Upper panel: CD spectra of TWJ without or with increasing amounts of ligands; lower panel: and UV-Vis spectra of corresponding concentrations of ligands.



CD-melting experiments. CD-melting experiments carried out with TWJ (1µM) was performed recording the CD signal at 278nm (the positive maximum of each CD spectrum) as a function of the temperature (from 25 to 85°C, bandwidth = 1nm, 1°C data pitch, 1s response). Experiments were carried without and with cryptands/TACN-Q (5µM) in 600µL (final volume) of 10mM lithium cacodylate buffer (pH 7.2).

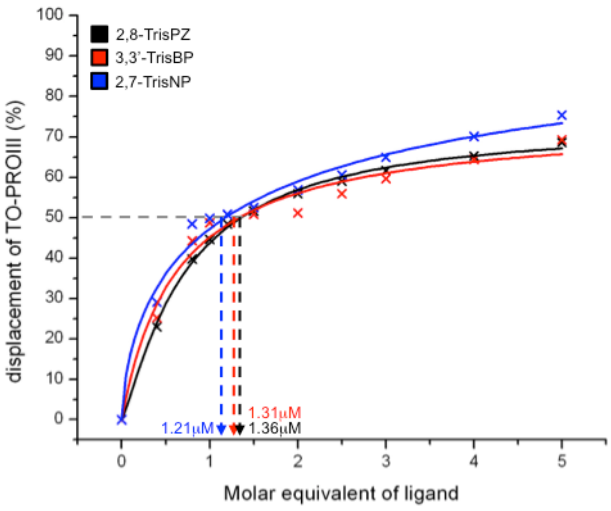
Part 6. Fluorescence and FID experiments.

TO-PRO-3 titrations. To better assess the interaction(s) that take(s) place between three-way DNA junction and the fluorescent dye TO-PRO-3 (Life Technologies), a first row of titration was performed, monitoring the evolution of the dye fluorescence as a function of its concentration in presence of a



fixed DNA concentration. To this end, a solution of TWJ (1μM) in 10mM lithium cacodylate buffer (pH 7.2) was added of increasing concentration (from 0 to 5μM) of TO-PRO-3 (1mM solution in DMSO); after a short equilibration time at 25°C (5-10min), the fluorescence spectra were recorded (λ_{ex} = 642nm, λ_{em} = 650-750nm). The fluorescence peak area was subsequently plotted as a function of the TO-PRO-3 molar equivalents; as seen in the figure on the left, the fluorescence intensity increases up to 1:1 dye:DNA ratio, is maximum at 1:1 ratio sharp, and progressively decreases at higher dye:DNA ratio (presumably due to the aggregation that originates in the self-association of free TO-PRO-3 in solution). This result clearly indicates that TWJ offers a unique, privileged binding site, presumably the cavity in light of previous reports dealing with cyanine dyes/three-way junction recognition (cf. M. N. Stojanovic & D. W. Landry, *J. Am. Chem. Soc.*, **2002**, *124*, 9678).

FID experiments. Fluorescence Intercalator Displacement assays were performed on a JASCO FP-8500 spectrofluorometer with TWJ (1μM) and TO-PRO-3 (1μM) in 1mL of 10mM lithium cacodylate buffer (pH 7.2). After a stabilization step (5-10min), the solutions were excited at 642nm and emission spectra were recorded between 650 and 750nm. Afterwards, ligands (2mM solution in water) were progressively added (from 0 to 5μM, *i.e.*, 0.4, 0.8, 1.0, 1.2, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0μM) and emissions were measured after fluorescence stabilization (5-10min at 25°C). The percentages of displacement were calculated from the variation of fluorescence area (FA) between 650-750nm, using: TO-PRO-3 displacement (%) = 100 - [(FA/FA_0) x 100], with FA_0 as the maximum fluorescence of TO-PRO-3 (bound to DNA and without ligand). The TO-PRO-3 displacement (%) was then plotted as a function of the concentration of added ligands.



Part 7. Cell culture and cell viability assay (MTT assay).

Cell culture. Briefly, the adherent B16 cell line was routinely cultured in 100x20mm tissue culture dish (Falcon) at 37°C in a humidified, 5% CO_2 atmosphere in Roswell Park Memorial Institute medium (RPMI 1640 (1X) Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% Penicillin-Streptomycin (Pen-Strep, 5.0u.mL^{-1} Pen/5.0μg.mL^{-1} Strep, Gibco) mixture. Cells were subcultured twice weekly using a standard protocol: the medium was first removed by aspiration; the cells were subsequently washed once with Dulbecco's Phosphate Buffered Saline (DPBS) and 1.5mL of a trypsin-EDTA solution (0.25%, Gibco) was added. After 5min at 37°C, cells were manually harvested and 500μL of the solution of cells in suspension was dispensed into three tissue culture dishes containing 10mL of RPMI medium (with 10% FBS and 1% Pen-Strep).

MTT assay. Cells were firstly subcultured for 24hrs into a 96-well plate (Microtest^{TM} 96, Falcon) prior to incubation with compounds. Stock solutions of each compound (2mM in H_2O) were firstly diluted in RPMI medium (with 10% FBS and 1% Pen-Strep) to 100μM final solutions. A two-fold serial dilution protocol (in a 1mL final volume) thus offered 8 concentrations (100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78μM) for each compound. Cells were subsequently incubated in presence of increasing concentrations (0 to 100μM) of ligands (2,7-TrisNP, 3,3'-TrisBP, 2,8-TrisPZ and TACN-Q), with 10 wells per line (8 different concentrations plus 2 control wells with RPMI buffer only), and 2 (duplicate) to 4 lines (quadruplicate) per compounds. After 48hrs, the medium was removed, cells were washed once with DPBS, and then incubated for 1hr at 37°C in a freshly prepared 2mg.mL^{-1} solution of MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide, Sigma-Aldrich) in DBPS. MTT solutions were delicately pipetted off and 100μL DMSO were added in each well (to dissolve the resulting blue formazan crystals, if any); after a gentle agitation for 1hr at 25°C, the lethal concentrations (LC_{50}) of each compound were evaluated by an optical density determination at 530nm.