

Electronic supplementary information

A FRET-enabled molecular peptide beacon with significant red shift for the ratiometric detection of nucleic acids

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1. Materials and devices:

Solvents were dried and distilled before use. Millipore water was obtained with a Micropure apparatus from TKA. Lyophilization was carried out with an Alpha 1-4 2D plus freeze drying apparatus from Christ. Analytical TLC was carried out on SiO₂ aluminium foils ALUGRAM SIL G/UV₂₅₄ from Macherey-Nagel. Reversed phase column chromatography was done with an Armen Instrument Spot Flash Liquid Chromatography MPLC apparatus with RediSep C-18 Reversed-Phase columns. The purity of the compounds was determined with the help of an HPLC apparatus from Dionex containing the following components: P680 HPLC pump, ASI-100 Automated Sample Injector and UVD 340U detector. A SupelcosilTM LC-18 column (25 cm × 4.6 mm, 5 μm) from Supelco or a YMC ODS-A column (15 cm × 3.0 mm, 5 μm) was utilized. Ultrapure water and HPLC-grade solvents were used as eluents. Detection was achieved with the help of a UV detector. ¹H- and ¹³C-NMR spectra were recorded on DRX 500 MHz spectrometer from Bruker at ambient temperature. The chemical shifts are reported in parts per million (ppm) relative to the deuterated solvent DMSO-d₆. All mass spectra were received by using a Bruker BioTOF III. Determination of pH values was carried out with a pH-Meter 766 Calimatic from Knick. Fluorescence spectra were obtained with a Varian Cary Eclipse spectrometer. Dynamic Light Scattering (DLS) was measured on Malvern Zetasizer nano zs with Dispersion Technology Software 5.03.

2. General procedure for the synthesis of the peptide:

Fmoc Removal: The Fmoc protecting group was cleavage by treatment with 20% piperidine in DMF (2×6 mL, 5 min each) under microwave radio condition (20 W, 50±5 °C, 5 min). Then, the resin was washed 3×8 mL with DMF, 3×8 mL with DCM, 3×8 mL with DMF (ca. 5 min each) to remove the last traces of piperidine. A positive Kaiser test confirmed the cleavage of the Fmoc group and the presence of free amino function.

Alloc deprotection: The Alloc protecting group was removed with Pd(PPh₃)₄ (0.1 eq) in the presence of PhSiH₃ (24 eq) in DCM for 20 min followed by washing with DCM (3× 5 mL) and DMF (3× 5 mL). A positive Kaiser test confirmed the cleavage of the Alloc group and the presence of free amino function.

Standard Fmoc solid phase peptide synthesis techniques (SPPS): Each amino acid was attached using 0.6 mmol/g loading Fmoc Rink amide resin under microwave radio (20 W, 60±5 °C, 20 min) Then, the resin was washed 3×8 mL with DMF, 3×8 mL with DCM, 3×8 mL with DMF (ca. 5 min each) to remove the last traces of the amino acid. A negative Kaiser test confirmed the attachment of the corresponding amino acid.

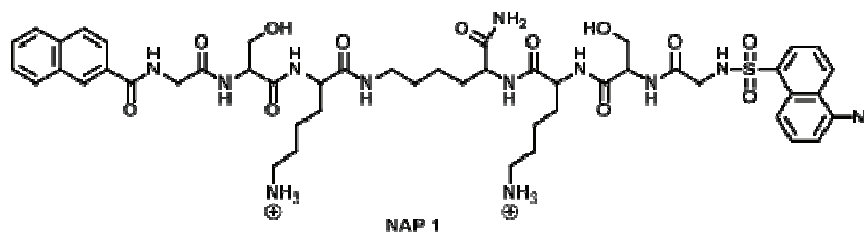
Cleavage from the Resin: Cleavage of the product from the resin was achieved by treatment with a mixture of TFA/H₂O/triisopropylsilane (95:2.5:2.5) for 3 h. The yellow cleavage mixture was collected by filtration and the resin was washed twice with pure TFA (6 mL). The filtrates were combined and concentrated under vacuum to obtain an oily residue. The peptide was precipitated by adding dry diethyl ether to the oil, following by centrifugation of the mixture. The precipitate was dissolved in water (25 mL), and the mixture was freeze-dried in vacuum. The resulting solid was purified by MPLC on C18 reversed phase silica gel (MeOH/water, 0.1 % TFA). Then the product was dissolved three times in HCl_{aq} (0.1 N) plus one time in water and consequently freeze-dried to obtain white solid.

Purity of the peptides was checked by HPLC on a RP18-column using water/MeOH (with 0.05% TFA) as solvent.

3. Synthesis of NAP 1 and characterization:

Rink amide resin (200 mg, 0.6 mmol/g, 1 equiv.) was weighed out into plastic peptide synthesis vessel and allowed to swell in DCM/DMF (5.0/5.0 mL) for 2 h. Then, the Fmoc protection group was removed by treatment with piperidine (20%) in DMF under microwave radio condition. After an intensive washing cycle with DMF the following four amino acid (Fmoc-Lys(Alloc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Gly-OH) and 1-naphthalenecarboxylic acid were attached under microwave condition for SPPS: Fmoc-protected amino acid (0.36 mmol, 3 equiv), PyBOP (0.36 mmol, 3 equiv) and DIPEA (0.72 mmol, 6 equiv) in DMF (8.0 mL). Then the Alloc protecting group was removed with Pd(PPh₃)₄ (0.012 mmol, 0.1 eq) in the presence of PhSiH₃ (2.88 mmol, 24 eq) in DCM followed by washing with DCM and DMF. Then three amino acid (Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Gly-OH) and dansylglycine were attached under similar microwave condition in DMF. Then, the resin was transferred into a glass peptide synthesis vessel and the product was cleaved from the solid support according to the general procedure for the Rink amide resin.

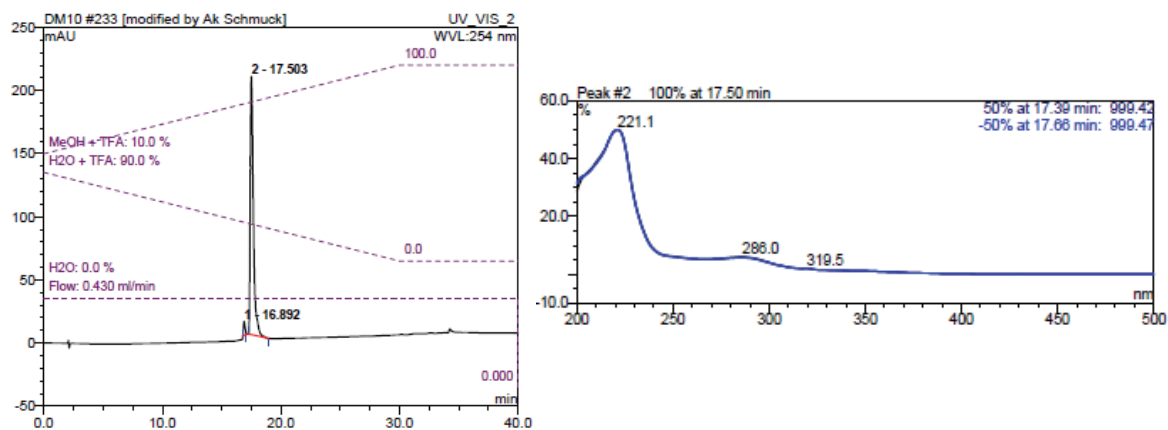
Peptide NAP 1:



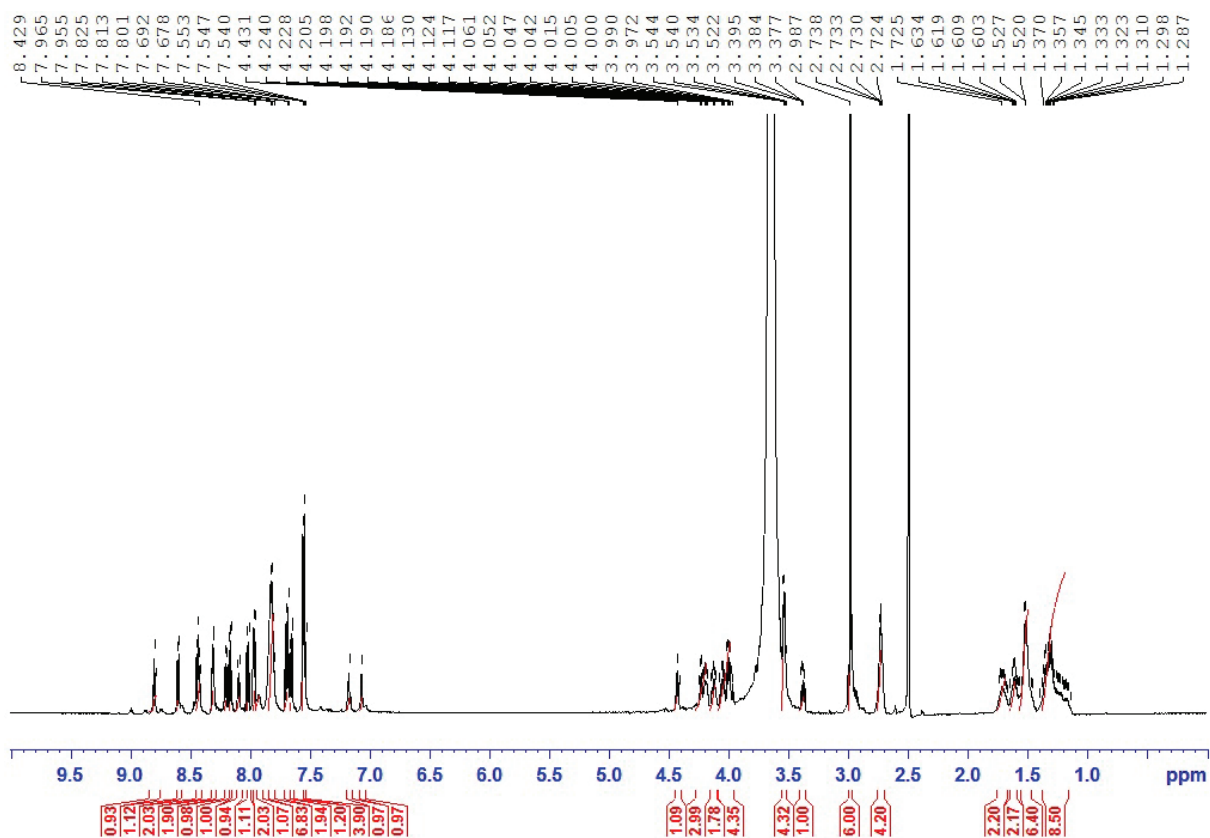
Peptide NAP 1 (31.0 mg, 28.7 μ mol, Yield 23.9%, purity HPLC 97%). ¹H NMR (500 MHz, DMSO-d₆): δ [PPM] 1.14-1.40 (m, 8H), 1.46-1.52 (m, 6H), 1.57-1.64 (m, 2H), 1.66-1.73 (m, 2H), 2.72 (m, 4H), 2.98 (s, 6H), 3.36-3.39 (dd, J = 5.2 Hz, 3.6 Hz, 1H), 3.52-3.54 (m, 4H), 3.96-4.07 (m, 4H), 4.10-4.14 (m, 2H), 4.17-4.25 (m, 3H), 4.40-4.44 (m, 1H), 7.06 (s, 1H), 7.16 (s, 1H), 7.52-7.55 (m, 4H), 7.64-7.70 (m, 3H), 7.79-7.82 (m, 7H), 7.91-7.97 (m, 3H), 8.00 (d, J = 6.9 Hz, 1H), 8.08 (d, J = 6.6 Hz, 1H), 8.15 (m, 1H), 8.18 (d, J = 6.2 Hz, 1H), 8.30 (m, 2H), 8.40-8.44 (m, 2H), 8.59 (d, J = 7.1 Hz, 1H), 8.79 (t, J = 4.8 Hz, 1H). ¹³C NMR (125.8 MHz, DMSO-d₆): δ [PPM] 22.26, 22.36, 22.46, 22.52, 22.95, 26.62, 28.59, 30.71, 31.01, 31.35, 31.63, 38.67, 38.74, 40.03, 41.15, 41.35, 42.75, 43.73, 43.85, 45.19, 45.89, 45.94, 51.26, 51.45, 52.82, 52.96, 53.14, 53.30, 54.84, 55.14, 61.82, 61.96, 62.72, 117.55, 122.67, 124.98, 125.18, 125.39, 125.53, 125.64, 125.78, 126.49, 126.91, 127.00, 127.76, 128.03, 128.33, 128.67, 128.88, 128.99, 129.86, 129.91, 130.23, 133.28, 134.11, 134.37,

136.27, 136.53, 146.32, 165.33, 165.63, 168.15, 169.40, 169.45, 170.08, 170.90, 171.35, 171.58, 174.08. FT-IR (pure): ν [cm⁻¹] 2928 1649 1519 1460 1322 1238 1140 1046 854 785 582. MALDI-TOF MS: calcd 1099.501 for C₅₁H₇₂N₁₂O₁₂S + Na⁺, found 1099.639.

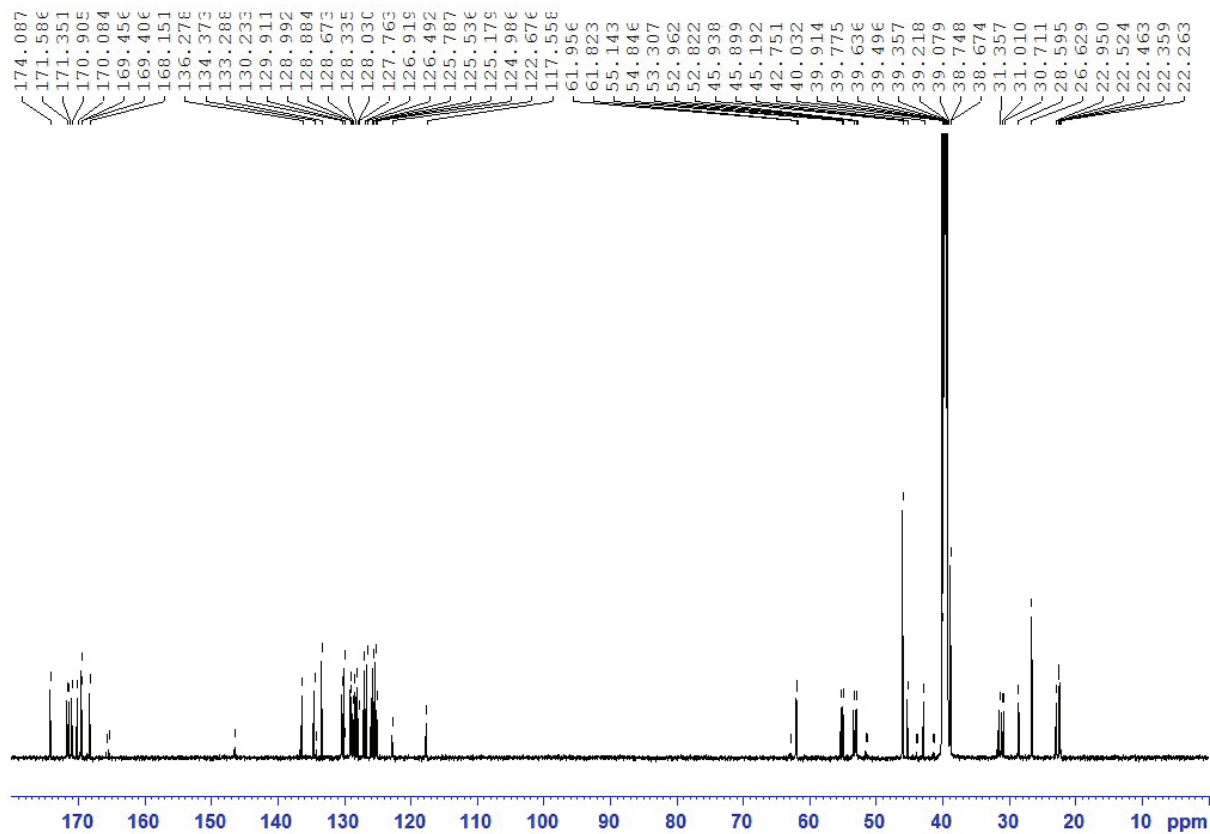
RP-HPLC chromatogram of NAP 1:



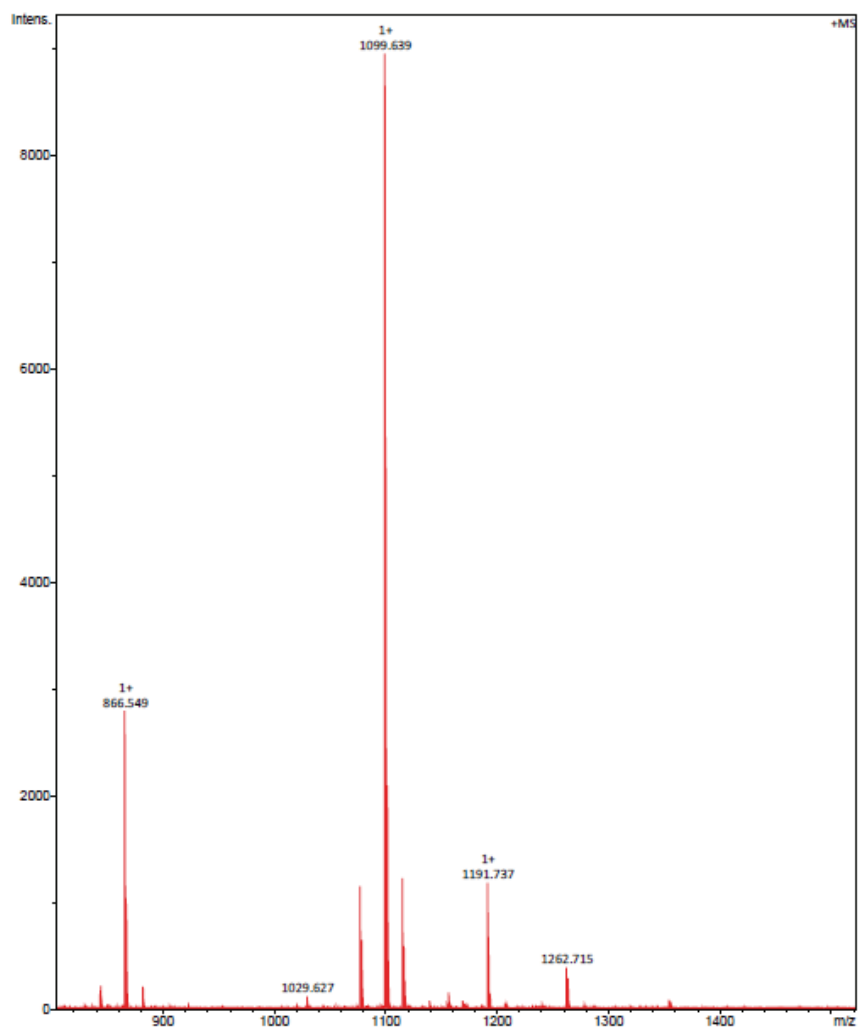
¹H NMR Spectra of NAP 1:



¹³C NMR Spectra of NAP 1:



MALDI-TOF MS spectra of NAP 1:



4. Fluorescence experiments:

General procedures: All fluorescence spectra were recorded using a VARIAN Fluorescence Spectrophotometer at 25 °C. 1 cm cells were used for emission titration. For fluorescence titrations stock solution of **NAP 1** was prepared ($c = 1000 \mu\text{M}$) in 10 mM HEPES buffer (pH = 7.4). The slit widths were set to 5 nm for excitation and emission. The data points were collected at 1 nm increments with a 0.1 s integration period. All spectra were corrected for intensity using the manufacturer-supplied correction factors and corrected for background fluorescence and absorption by subtracting a blank scan of the buffer system.

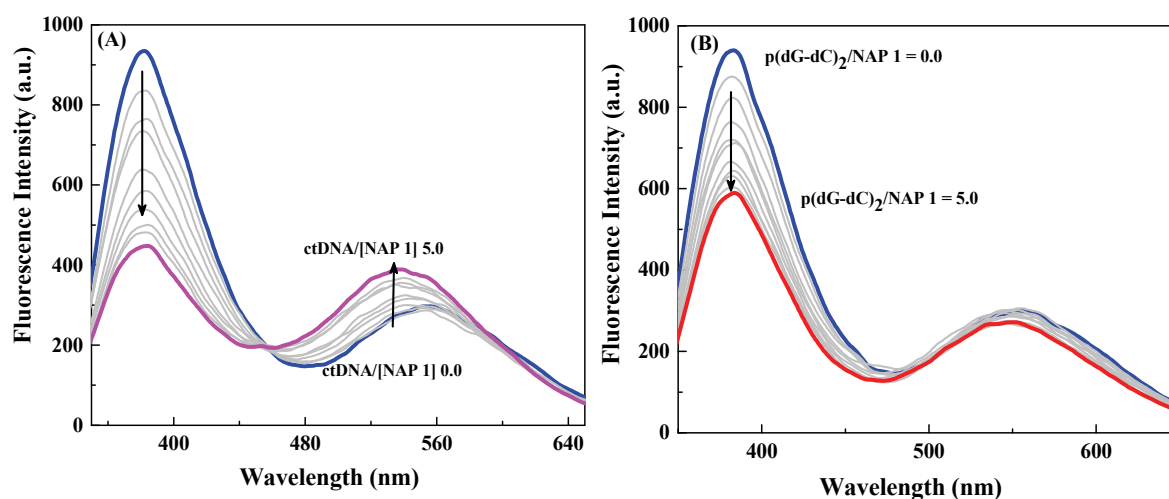


Fig. S1 Fluorescence emission spectra for the titration of a 10 μM solution of **NAP 1** with (A) ct-DNA and (B) p(dG·dC)₂ at 25 °C in 10 mM HEPES buffer (pH 7.4) (with base pair/**NAP 1** molar ratios ranging from 0 to 5.0) ($\lambda_{\text{ex}} = 310 \text{ nm}$).

5. DAPI displacement assay:

First, a solution of DAPI (4', 6-Diamidino-2-phenylindole) was prepared in 10 mM HEPES buffer (pH 7.4) in a fluorescent microcuvette with a stopper (950 μL) at a concentration of 1.0 μM (1.0 eq.). Upon excitation at 340 nm the fluorescence spectrum between 400 and 600 nm was recorded at 25 $^{\circ}\text{C}$. Then the polynucleotide was added (4.0 μM , 4.0 eq.) and after 5 min incubation time the spectrum was measured again, now with increased fluorescence intensity due to the minor groove binding of DAPI. Stock solutions of **NAP 1** (1.0 mM) were added to the mixture in aliquots of increasing volume (e.g. 0.4 μL) and the spectrum was measured after 5.0 min incubation time after each addition. **Fig. S2** depicts a titration with p(dA•dT)₂ and the excerpt at 460 nm, which was corrected for dilution and DAPI's own emission before fitting with a first order exponential decay function. The average amounts of **NAP 1** which were necessary to displace 50% of DAPI were calculated. By carrying out additional titrations of DAPI into a solution of **NAP 1** it was ensured that there is no interaction between these molecules.

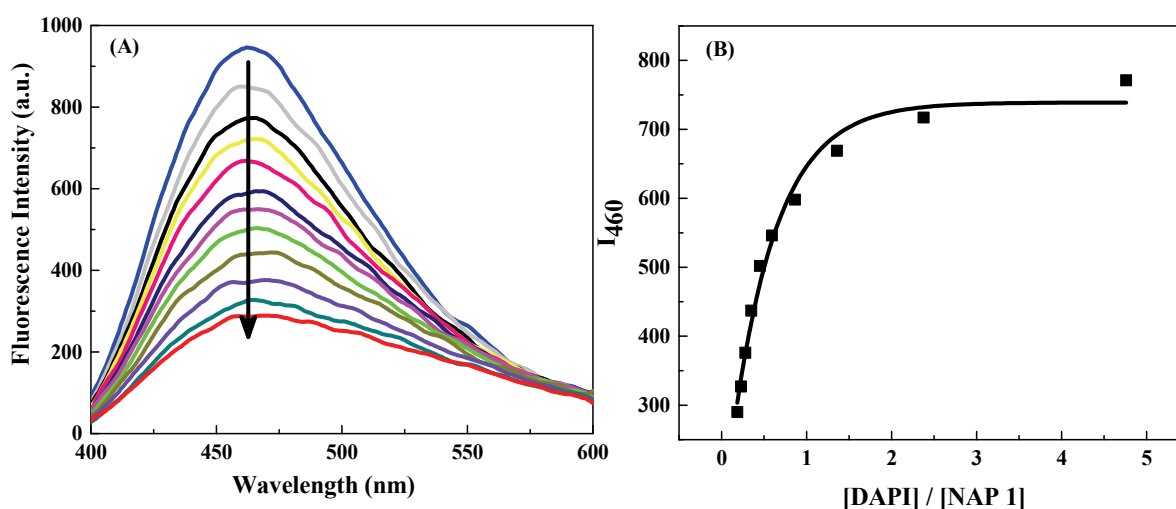


Fig. S2 (A) DAPI displacement experiment with **NAP 1**-p(dA•dT)₂ 10 mM HEPES buffer (pH 7.4); (B) excerpt of the fluorescence emission at 460 nm plotted against the ratio of DAPI, $\text{IC}_{50} = 2.25 \mu\text{M}$ (p(dA•dT)₂).

6. Cell image experiments:

Cell imaging: A549 cells were seeded in a glass bottom dish and allowed to adhere for 24 h. The cells were washed and incubated with **NAP 1** (6 μ M) in RPMI 1640 medium respectively for 45 min at 37°C. Cell imaging was carried out after washing the cells with phosphate buffered saline (PBS, pH 7.4).

Co-localization imaging of fixed cells: A549 cells were seeded in a glass bottom dish and allowed to adhere for 24 h. The cells were incubated with DAPI (1 μ g/mL) in RPMI 1640 medium for 6 h at 37°C with 5% CO₂. After washing the cells with PBS (pH 7.4), the cells were washed and incubated with **NAP 1** (6 μ M) in RPMI 1640 medium respectively for 30 min at 37°C. Cell imaging was then carried out after washing the cells with PBS (pH 7.4) with confocal laser scanning microscope Cell images were obtained with a confocal laser scanning microscope (CLSM), Nikon A1 (Japan), at an excitation wavelength of 405 nm and the emission was collected at 424-454 nm and 534-594 nm.

7. Cytotoxicity assay:

MTT assay: The cytotoxicity was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay with A549 cell line. Cells growing in log phase were seeded into 96-well cell-culture plate at a density of 1×10^4 cells/well and incubated overnight at 37 °C under 5% CO₂. After the complete medium was removed, the sample solution of **NAP 1** (100 µL/well) at concentrations of 0.5, 1, 2, 4, 6, 8 µM in RPMI-1640 medium was added to the wells of the treatment group, respectively, whereas for final negative control group 100 µL of RPMI-1640 was added, respectively. The cells were incubated for 24 h at 37 °C under 5% CO₂. After removal of the medium, 10 µL of MTT solution (5 mg/mL in PBS) was then added to the plates for additional 4 h incubation, allowing viable cells to reduce the yellow tetrazolium salt (MTT) into dark blue formazan crystals. After removal of the medium, formazan extraction was solubilized with 100 µL DMSO and its quantity determined colorimetrically using a Synergy H4 Hybrid Microplate reader (Biotek, USA) at 490 nm (Absorbance value). Cell viability (%) was calculated using the following formula: Cell viability (%) = (mean of Absorbance value of treatment group/mean Absorbance value of control-blank) × 100.

8. Molecular modeling studies

Virtual molecular studies were carried out by a conformational search using Macro Model^[1] with the OPLS2005 forcefield, GB/SA solvent model for water, 500000 iterations and a convergence threshold of 0.05. The conformational search has been processed with mixed torsional / low-mode sampling with 1000 maximum steps and 100 steps per rotatable bond. The collected conformations have been in an energy window of 5 kJ/mol and have been of similar shape, so that the shown structure (fig. 2) represents all found conformations in that specific energy window. PyMOL^[2] was used for illustration.

The simulation was done on different initial positions of NAP-1 at the DNA, to receive information about possible binding conformations at the different grooves (minor, major and mixed grooves). For a better comparison of energies, the energy of the most stable variant was subtracted from all energies to obtain the following table.

Table S1 Relative energies of NAP-1 at the different sites.

Binding Site	Energy [kJ/mol]	Δ Energy [kJ/mol]
Minor Groove	-68641.789	0
Mixed Grooves	-68563.711	78.078
Major Groove	-68534.719	107.07

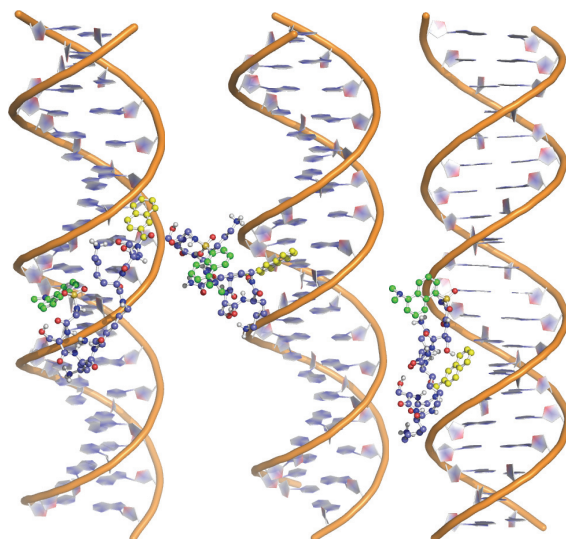


Fig. S3 Conformations of **NAP-1** at the different sites. (Dansyl colored in green and Naphtyl colored in yellow) From left to right: Conformation at minor, mixed or major groove, respectively.

[1] MacroModel, version 9.9, Schrödinger, LLC, New York, NY, 2012.

[2] The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.