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Supporting Information

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for

Efficient DNA Binding and Nuclear Uptake by Distamycin Derivatives Conjugated to Octaarginine Sequences

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Abbreviations

HBTU	*o*-(1-benzotriazol-1-*yl*)-1,1,3,3-tetramethyluronium hexafluorophosphate
HATU	*o*-(7-azabenzotriazol-1-*yl*)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt	1-hydroxybenzotriazole
TFA	trifluoroacetic acid
DIEA	*N,N*-diisopropylethylamine
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4',6'-diamidino-2-phenylindole
MWD	Multiple Wavelength Detector
RP	Reverse Phase
HPLC	High-Performance Liquid Chromatography
LC/MSD	Liquid Chromatograph Mass Spectrometer Detector
TNBS	2,4,6-Trinitrobenzene sulfonic acid
TIS	Triisopropylsilane
FITC	Fluorescein isothiocyanate
DMF	Dimethylformamide
Alloc	Allyloxycarbonyl
DEDTC	Diethyldithiocarbamate
EDT	Ethane-1,2-dithiol
DMAP	4-Dimethylaminopyridine
DTNB	5,5'- Dithiobis-2-nitrobenzoic acid
PAGE	Polyacrylamide gel electrophoresis
TBE	Tris-Borato-Ethylen-diamine tetra-acetic acid
DMEM	Dulbecco Modified Eagle Medium
FBS	Fetal Bovine Serum
PBS	Phosphate Buffered Saline
Pip	Piperidine
Ahx	6- Aminohexanoic acid

Materials

All reagents were acquired from commercial sources: HBTU, HATU, HOBt and Fmoc-protected amino acids were purchased from GL Biochem (Shanghai), except for the orthogonally protected Fmoc-Lys(Alloc)-OH which was purchased from Bachem. DMF was adquired from Scharlau (peptide synthesis grade), CH_2Cl_2 from Panreac, TFA from SDS, CH_3CN from Merck, DIEA from Fluka, DMEM and FBS from Invitrogen and SyBrGold® from Molecular Probes. The rest of reagents were acquired from Sigma-Aldrich.

UV Spectroscopy

The molar extinction coefficient used to measure the concetration of the compounds containing fluoresceine was ε_{494nm}= 77 000 cm^{-1} M^{-1} at pH 9. The molar extinction coefficient used for those compounds that only have the trypirrol as chromophore was ε_{304nm}= 32 274 cm^{-1} M^{-1} while for those including the chromophore ABA (*p*-acetamido benzoic acid) we used $\varepsilon_{270\,nm}$ =18 069 cm^{-1} M^{-1}. In the case of the oligonucleotides, molar extinction coefficient were determinated for each single strand by using the following formula:[1]

$$\varepsilon_{260\,nm} = \{(8.8 \times \#T) + (7.3 \times \#C) + (11.7 \times \#G) + (15.4 \times \#A)\} \times 0.9 \times 10^{3}\ M^{-1}cm^{-1}$$

Analytical data of the purified products 1 A,B – 4 A,B

The purification of all compunds was performed by preparative HPLC, using an isocratic regime during the first 5 min, followed by a linear gradient from 5% to 75% of solvent B for 30 min (A: water with 0.1% TFA, B: acetonitrile with 0.1% TFA).

1A ~ 26% yield, considering peptide synthesis: MS: calcd. for $C_{120}H_{193}N_{47}O_{22}S$ = 2675.2 $[M+H]^{+}$; found MS (ESI): 892.5 $[M+H_3]^{3+}$ (7), 669.6 $[M+H_4]^{4+}$ (30), 535.9 $[M+H_5]^{5+}$ (75), 446.7 $[M+H_6]^{6+}$ (30).

2A ~20% yield, considering peptide synthesis: MS: calcd. for $C_{119}H_{180}N_{34}O_{26}S$ = 2534.3 $[M+H]^{+}$; found MS (ESI): 845.1 $[M+H_3]^{3+}$ (100), 633.9 $[M+H_4]^{4+}$ (33), 507.2 $[M+H_5]^{5+}$ (5), 422.6 $[M+H_6]^{6+}$ (5), 317.7 $[M+H_8]^{8+}$ (25).

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[1] Sambrook, J.; Fritsch, E. F.; and Maniatis, T. *Molecular Cloning, A Laboratory Manual*, 2nd ed, p. 11.30. **1989** Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

3A ~17% yield, considering peptide synthesis: MS: calcd. for $C_{116}H_{173}N_{30}O_{27}S$ = 2449.3 $[M+H]^+$; found MS (ESI): 1225.6 $[M+H_2]^{2+}$ (20), 817.1 $[M+H_3]^{3+}$ (47), 613.8 $[M+H_4]^{4+}$ (25). t_R= 22.4 min.

4A ~20% yield, considering peptide synthesis: MS: calcd. for $C_{169}H_{279}N_{66}O_{36}S$ = 3841.2 $[M+H]^+$; found MS (ESI): 961.3 $[M+H_4]^{+4}$ (5), 769.2 $[M+H_5]^{+5}$ (15), 641.2 $[M+H_6]^{6+}$ (40). t_R= 21.3 min.

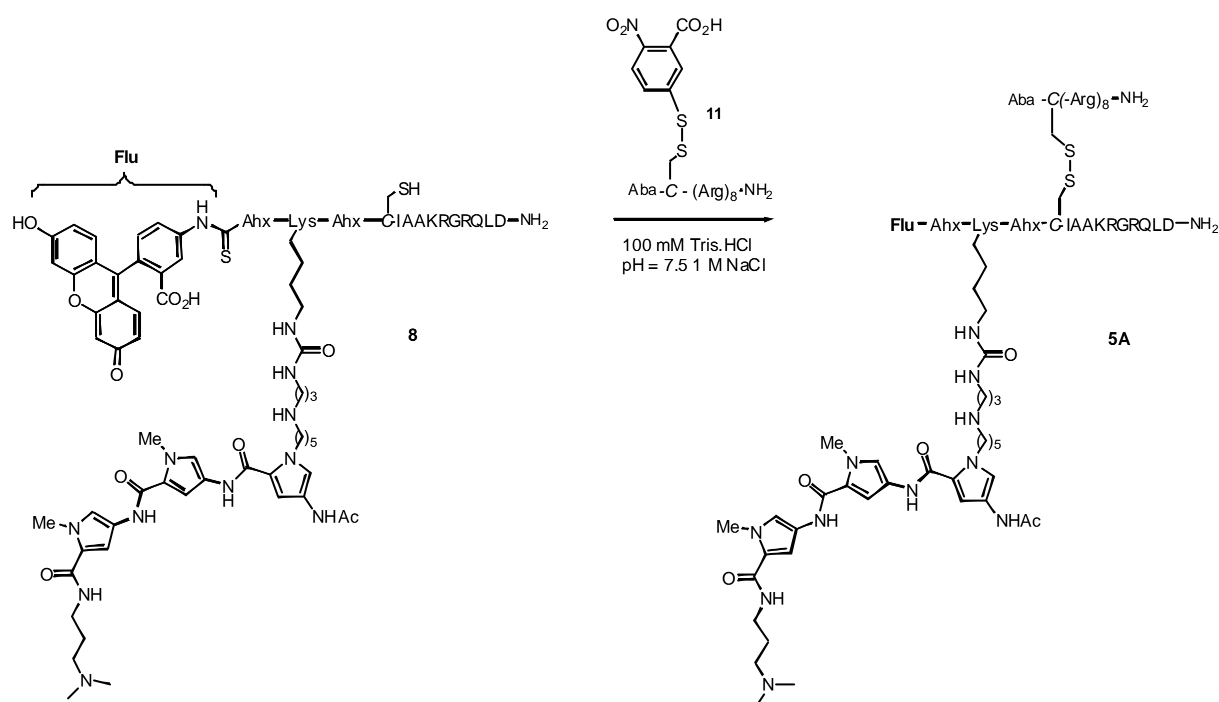
1B ~ 90% yield, considering peptide synthesis: MS:$^+$: calcd. for $C_{87}H_{143}N_{37}O_{17}S$ = 2010.1 $[M+H]$; found MS (ESI): 670.8 $[M+H_3]^{+3}$ (80), 503.5 $[M+H_4]^{4+}$ (50), 402.9 $[M+H_5]^{+5}$ (25), 335.8 $[M+H_6]^{+6}$ (10). t_R= 16.2 min.

2B ~ 70% yield, considering peptide synthesis: MS: calcd. for $C_{86}H_{131}N_{23}O_{22}S$ = 1870.0 $[M+H]^+$; found MS (ESI): 935.5 $[M+H_2]^{+2}$ (35), 624.0 $[M+H_3]^{3+}$ (100), 468.4 $[M+H_4]^{+4}$ (40), 374.9 $[M+H_5]^{+5}$ (5). t_R= 21.1 min.

3B ~ 80% yield, considering peptide synthesis: MS: calcd. for $C_{83}H_{125}N_{20}O_{22}S$ = 1785.9 $[M+H]^+$; found MS (ESI): 893.0 $[M+H_2]^{+2}$ (70), 595.8 $[M+H_3]^{3+}$ (100), 447.0 $[M+H_4]^{+4}$ (20). t_R= 21.7 min.

4B ~ 83% yield, considering peptide synthesis:MS: calcd. for $C_{136}H_{231}N_{56}O_{31}S$ = 3176.8 $[M+H]^+$; found MS (ESI): 795.9 $[M+H_4]^{+4}$ (30), 636.2 $[M+H_5]^{+5}$ (60), 530.4 $[M+H_6]^{+6}$ (100), 454.3 $[M+H_7]^{+7}$ (40). t_R= 20.7 min.

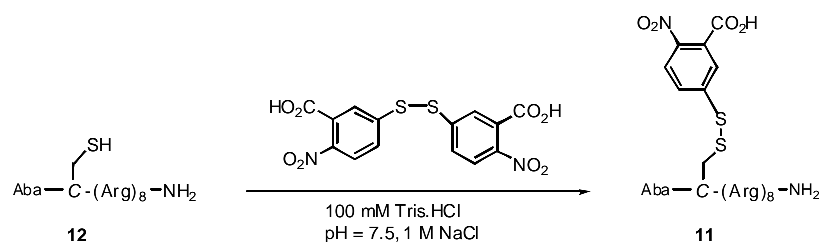
Synthesis of the disulfide derivate ***5A***



Scheme S1.

To a solution of peptide **8** (2.2 mg, 8.3× 10^{-4} mmol) in a mixture of desoxygenated CH_3CN (200 µL) and a buffer composed by 100 mM TrisHCl pH 7.5 and NaCl (1 M, 350 µL), was added a desoxygenated solution of the activated peptide **11** (2 mg, 1.2 × 10^{-3} mmol) in the same buffer (80 µL). The mixture was stirred at room temperature for 30 min and the reaction was monitored by HPLC. The mayor product was purified by RP-HPLC and identified by as the activated peptide **5A** (1.4 mg, 40%): MS: calcd. for $C_{182}H_{293}N_{68}O_{40}S_3$ = 4166.2 $[M+H]^+$; found MS (ESI): 1466.1 $[M+H_5(CF_3CO_2)_2]^{+3}$ (20), 1071.4 $[M+H_5(CF_3CO_2)]^{4+}$ (65), 940.0 $[M+H_4]^{+4}$ (25), 1042.5 $[M+H_4]^{+4}$ (100), 900.7 $[M+H_8(CF_3CO_2)_3]^{+5}$ (40), 639.4 $[M+H_9(CF_3CO_2)_2]^{+7}$ (75). t_R = 20.7.

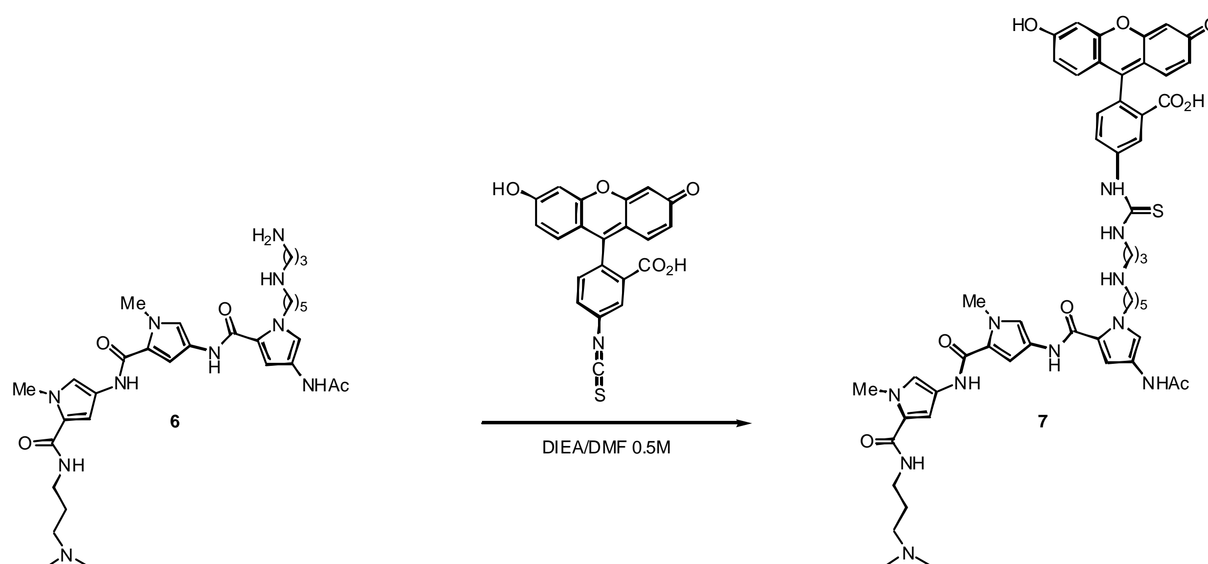
Preparation of peptide **11**.



Scheme S2.

A solution of DTNB (5,5'- Dithiobis-2-nitrobenzoic acid, Ellman's reagent) (3.1 mg, 7.8×10^{-3} mmol) in a mixture of desoxygenated CH_3CN (25 µL) and a buffer composed by 100 mM TrisHCl pH 7.5 and 1 M NaCl (100 µL) was added to desoxygenated solution of the peptide **12** (6 mg, 3.9×10^{-3} mmol) in the same buffer (200 µL). The mixture was stirred at room temperature for 45 min and the reaction was monitored by HPLC. The mayor product was purified by RP-HPLC and identified as the activated peptide **11** (5 mg, 74%) MS: $[M+H]^+$: calcd. for $C_{67}H_{114}N_{35}O_{16}S_2$ = 1728.9; found MS(ESI): 865.2 $[M+H_2]^{2+}$, 576.8 $[M+H_3]^{3+}$.

Fluorescent Labelling of the aminotripyrrole **6**.

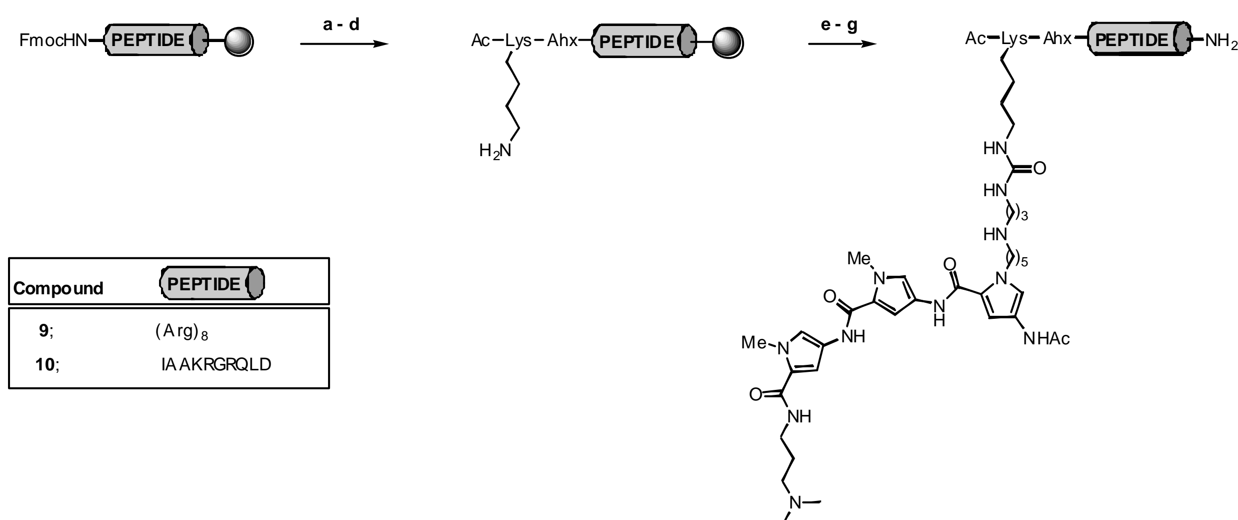


Scheme S3.

To a solution of aminotripyrrole **6**[2] (5.5 mg, 8.6×10^{-3} mmol) in DIEA (200 µL, 0.5 M in DMF) was added a solution of fluorescein isothiocyanate (1.68 mg, 4.3×10^{-3} mmol in 140 µL of DMF). The mixture was stirred for 2 h and the major product was purified by RP-HPLC and identified as the desired pyrrole **7** (1.7 mg. 39%) MS: $[M+H]^+$: calcd. for $C_{53}H_{62}N_{11}O_9S$ = 1028.4; found MS(ESI): 1028.4 $[M+H]^+$

[2] M.E. Vázquez, A.M. Caamaño, J. Martínez-Costas, L. Castedo, J.L. Mascareñas, *Angew. Chem. Int. Ed.* **2001**, *40*, 4723-4725; J.B. Blanco, M.E. Vázquez, L. Castedo, J.L. Mascareñas, J. L. *ChemBiochem.* **2005**, *6*, 2173-2177.

General synthetic procedure for the preparation of conjugates **9** *and* **10** *(analogues of* **1A** *and* **2A** *that lack the fluorescein label).*



Compound	PEPTIDE
9;	$(Arg)_8$
10;	IAAKRGRQLD

Scheme S4. a) i. Pip/DMF 20%; ii. HOBT/HBTU, DIEA/DMF, Fmoc- Ahx- OH; b) i. Pip/DMF 20%; ii. HOBT/HBTU, DIEA/DMF, Fmoc- Lys(Alloc)- OH; c) Ac_2O/DMF 20% and DIEA in DMF(0.195 M); d) $[Pd(PPh_3)_4]$ (1 equiv), morpholine (190 equiv), 2% H_2O/CH_2Cl_2, 5 h; e) DIEA/DMF 0.5 M, DMAP/DMF, N,N'-Disuccinimidyl carbonate; f) DIEA/DMF (0.5 M) and the aminotripyrrol **7**; g) 90% TFA, 50% CH_2Cl_2, 2.5% H_2O and 2.5% TIS, RT. Pip = piperidine, Ahx = 6- aminohexanoic acid, DIEA = N,N-diisopropylethylamine.

Removal of Fmoc group on the peptide was carried out following standard solid-phase methods condictions. The Fmoc-6-aminohexanoic acid was activated and coupled to the peptide under standard conditions. The acetylation was performed by adding a 500 µL aliquot of acetic anhydride in DMF (20%) and 250 µL aliquot of DIEA in DMF (0.195M) to the resin-bound free amino-terminal peptides. Nitrogen was passed through the mixture for 30 min. Then, the resin was filtered and washed with DMF (3 × 1.5mL × 3min) and CH_2Cl_2 (2 × 1.5mL × 3min) and dried under vacuum. The rest of the steps (d-g) have been explained before in material and methods section of the paper. Purification by preparative HPLC led to the desired products.

9, 30% yield, considering peptide synthesis: MS: exact mass calcd. for $C_{95}H_{172}N_{45}O_{17}$ = 2215.4 $[M+H]^+$; found MS (ESI): 739.5 $[M+H_3]^{3+}$, 554.6 $[M+H_4]^{4+}$, 669.6 $[M+H_4]^{4+}$, 443.9 $[M+H_5]^{5+}$.

10, 25% yield, considering peptide synthesis, MS: exact mass calcd. for $C_{94}H_{160}N_{31}$-O_{22} = 2074.2 $[M+H]^{+}$; found MS (ESI): 1038.3 $[M+H_2]^{2+}$, 692.3 $[M+H_3]^{3+}$, 519.5 $[M+H_4]^{4+}$, 416.0 $[M+H_5]^{5}$.

Additional results of cell internalization studies

As shown in Figure S1, compound **1A** presents an intense fluorescence in the nucleus even after only 30 min.

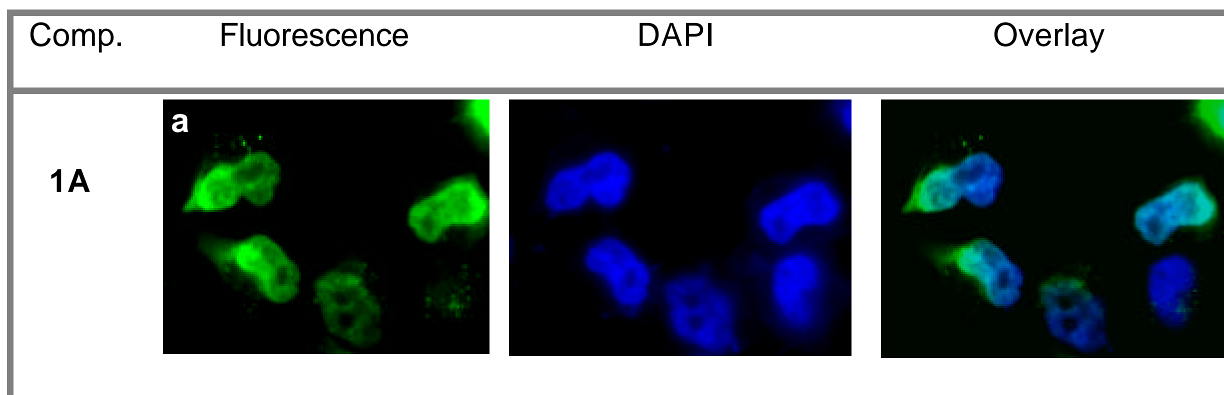


Figure S1. Intracellular distribution of the **1A** in HeLa cells after 30 min of incubation. The exposure times were: 1/11.

Bright field images of HeLa cells allow to verify the proper cell morphology.

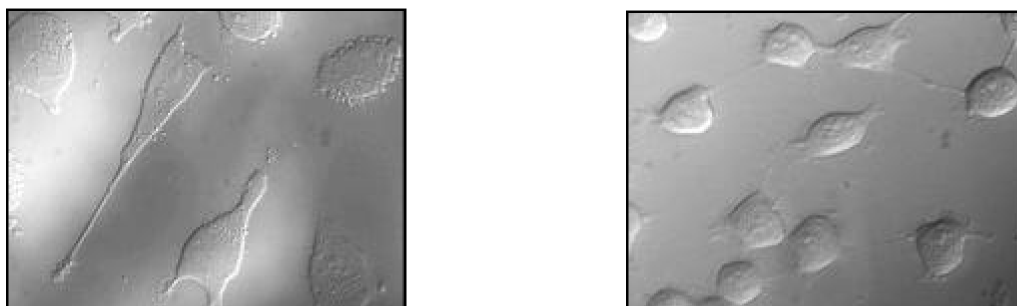


Figure S2. Bright field images of HeLa cells used in the experiments.

Gel mobility shift assays

Titration of the conjugate **9** for dsDNA *ATTTT* = 5'-GAGG*ATTTT*CAGCTTACGCT-3', led to a K_d = 6 ± 0.5 nM at 22 °C.

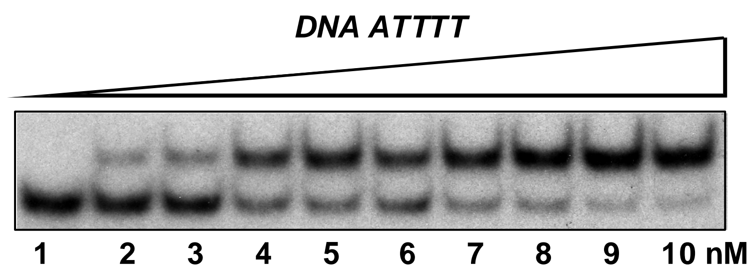


Figure S*3*. EMSA results showing the binding of peptides **9** to dsDNA *ATTTT*. The experiment was carried out with 45 pM of P^{32}DNA, lanes 1- 10: [**9**]= 0, 2, 4, 6, 8, 10, 12, 15, 20, 25 nM.

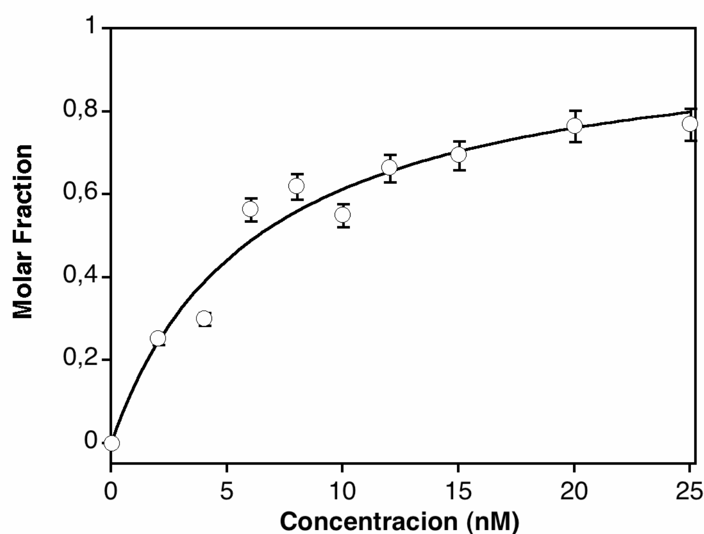


Figure S*4*. Binding isotherm resulting from densitometry analysis of gel in Figure S2. The curve represents the best fit to the data using nonlinear analysis with the Kaleidagraph 3.6 program (Synergy Software) to the equation derived using a 1:1 model.[3]

[3] M. H. A. Roehrl, J. Y. Wang, G. Wagner *Biochemistry* **2004**, *43*, 16056-16066.