Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supplementary Information

Sequence-Selective DNA Binding with Cell-Permeable Oligoguanidinium-Peptide Conjugates

Jesús Mosquera,[a] Mateo I. Sánchez,[a] Julián Valero,[b] Javier de Mendoza,[b] M. Eugenio Vázquez,[a] José L. Mascareñas[a].

[a]*Departamento de Química Orgánica e Centro Singular de Investigación en Química Biolóxica e Materiais Moleculares (CIQUS). Universidade de Santiago de Compostela. 15782 Santiago de Compostela (Spain).*

[b] Institute of Chemical Research of Catalonia (ICIQ), Avda. Països Catalans 16, 43007 Tarragona, Spain.

E-mail: eugenio.vazquez@usc.es, joseluis.mascarenas@usc.es

General

All reagents were acquired from commercial sources: DMF and TFA were purchased from *Scharlau*, CH_2Cl_2 from *Panreac*, CH_3CN from *Merck*. The rest of reagents were acquired from *Sigma-Aldrich*. All peptide synthesis reagents and amino acid derivatives were purchased from *GL Biochem* (Shanghai) Ltd. and *NovaBiochem*. Amino acids were purchased as protected Fmoc amino acids with the standard side chain protecting groups. Chromophore *p*-ABA (4-acetamidobenzoic acid) was purchased from *Sigma-Aldrich*.

Reactions were followed by analytical RP-HPLC with an *Agilent 1100* series LC/MS using an *Eclipse XDB-C18* analytical column (4.6 × 150 mm, 5 µm). Electrospray Ionization Mass Spectrometry (ESI/MS) was performed with an *Agilent 1100* Series LC/MSD model in positive scan mode using direct injection of the purified peptide solution into the MS. Standard conditions for analytical RP-HPLC consisted on an isocratic regime during the first 5 min, followed by a linear gradient from 5% to 75% of solvent B for 30 min at a flow rate of 1 mL/min (A: water with 0.1% TFA, B: acetonitrile with 0.1% TFA), compounds **1** and **2** were analyzed in the same equipment but with a linear gradient 5 to 95% of B en 30 min.. Compounds were detected by UV absorption. Purification were performed by semipreparative reverse-phase (RP) HPLC with an *Agilent* 1100 series LC using a *Luna* 5u C18(2) 100A (5 µm, 10 × 250 mm) reverse-phase column from *Phenomenex.* Concentrations were measured using the listed extinction coefficients.

Oligonucleotides were purchased from *Thermo Fisher Scientific* GmbH on a 0.2 mmol scale as freeze-dried solids. After solving in H_2O *milliQ* their concentrations were measured by UV absorption at 260 nm with a *BioRad* SmartSpec Plus Spectrophotometer. Absorbance was measured twice and concentrations were calculated applying Lambert-Beer's equation. The molar extinction coefficients of single strand oligonucleotides were calculated by using the following formula,[1]

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

Where #A, #T, #C, #G stand for the number of each type of bases in the DNA strand. Oligonucleotides were hybridized by mixing complementary sequences at equal molar concentration, heating at 90 °C for 10 min and then slowly cooling the mixture to rt over 1h.

dsDNAs used in this article (only one strand is shown):

AP1^{hs}•A/T = 5'-ACGAACG ***TCAT*AATTT**CCTC-3'
AP1^{hs}•G·A/T = 5´-ACGACG***TCAT*GAATTT**CCTC-3´
AP1^{hs}•GGG·A/T 5´-AGACG***TCAT*GGGAATTT**CCTC-3´
mAP1^{hs}•A/T = 5'-ACGAACG **CGGCAATTT**CCTC-3'
AP1^{hs}•mA/T = 5'-ACGAACG ***TCAT*GGCCG**CCTC-3'
A/T = 5'-CGCG **AATT** CGCG-3'
mA/T = 5'-CGCG **CATG** CGCG-3'
G/C = 5'-CGCG **GGCC** CGCG-3'

1 a) K. C. Engman, P. Sandin, S. Osborne, T. Brown, M. Billeter, P. Lincoln, B. Nordén, B. Albinsson, L. M. Wilhelmsson. *Nucl. Acids Res.* **2004**, *17*, 5087; b) G. Kallansrud, B. Ward. *Anal Biochem*. **1996**, *236*, 134.

Compound **1**, **2** and **3** were synthesized following known protocols.[2]

Synthesis

C-terminal amide peptides were synthesized following standard peptide protocols (Fmoc/tBu strategy) on a 0.1 mmol scale using a 0.19 mmol/g loading Fmoc-PAL-PEG-PS resin from Applied Biosystems, using a PS3 automatic peptide synthesizer from Protein Tecnologies. The amino acids were coupled in 4-fold excess using HBTU as activating agent. Each amino acid was activated for 30 seconds in DMF before being added onto the resin, and couplings were conducted for 30 min. Deprotection of the temporal Fmoc protecting group was performed by treating the resin with 20% piperidine in DMF for 10 min. Manual couplings were monitored using the TNBS (trinitrobenzene sulfonate) test.[3]

The resin cleavage-deprotection process was accomplished by stirring the resin-bound peptides (approx. 0.025 mmol), in 3 mL of the cleavage cocktail S (25 μL of EDT (1,2-ethanedithiol), 25 μL of H_2O, 10 μL of TIS (triisopropylsilane) and 940 μL of TFA). The resulting suspension is shaken for 2 h. The resin is filtered, and the TFA filtrate was added to ice-cold diethyl ether (30 mL). After 10 min, the precipitate was centrifuged and washed again with 20 mL of ice-cold ether. The solid residue is dried under argon, redissolved in acetonitrile/water 1:2 (2 mL) and purified by semipreparative RP-HPLC. The collected fractions are lyophilized and stored at −20 °C. The identity of the peptides (white powders) was confirmed by ESI^+-MS and MALDI-TOF.

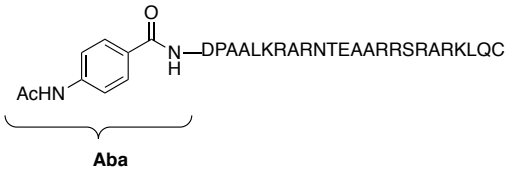
The resin cleavage-deprotection was accomplished by shaking the resin-bound peptides for 2 h (approx. 0.025 mmol), in 3 mL of the cleavage cocktail cleavage cocktail S (25 μL of EDT (1,2-ethanedithiol), 25 μL of H_2O, 10 μL of TIS (triisopropylsilane) and 940 μL of TFA). The resin is filtered, and the TFA filtrate is added to ice-cold diethyl ether (30 mL). After 10 min, the precipitate is centrifuged and washed again with 20 mL of ice-cold ether. The solid residue is dried under argon, dissolved in acetonitrile/water 1:1 (2 mL) and purified by semipreparative RP-HPLC. The collected fractions are lyophilized and stored at –20 °C.

2 *a*) J. Fernández-Carneado, M. V. Gool, V. Martos, S. Castel, P. Prados, J. de Mendoza and E. Giralt, *J. Am. Chem. Soc.* **2005**, *127*, 869; *b*) J. Valero, M. V. Gool, R. Pérez-Fernández, P. Castreño, J. Sánchez-Quesada, P. Pradosb and J. de Mendoza, *Org. Biomol. Chem*. **2012**, *10*, 5417.

3 W.S. Hancock, J.E. Battersby *Anal. Biochem.* **1976**, *71*, 260.

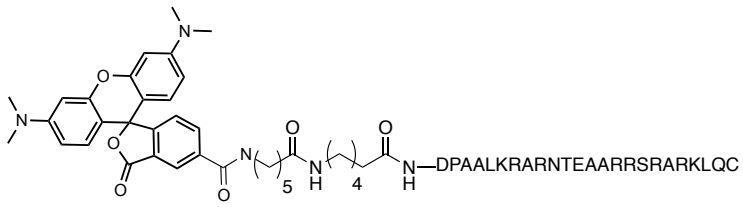
***Synthesis of* brC**

The peptide **brC** was prepared by the previously described SPPS procedures using 520 mg of Fmoc-Pal-PEG-PS (0.1 mmol). Once removed the Fmoc group of the last amino acid, the *Aba* chromophore was coupled to the *N*-terminus using 4 equiv of HATU, another 4 equiv of 4-acetomidobenzoic acid and 6 equiv DIEA 0.2 M in DMF for 30 min. The deprotection step were carried out following standard conditions described before, the resulting crude mixtures were purified by RP-HPLC obtaining the desired peptides on yields between 30-50%.



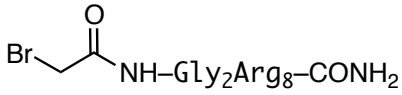
***Synthesis of* TMR–brC**

The peptide **TMR-brC** was prepared by the previously described SPPS procedures using 260 mg of Fmoc-Pal-PEG-PS (0.05 mmol). Once removed the Fmoc group of the last amino acid, two units of 6-(Fmoc-amino) caproic acid were coupled at the *N*-terminus residue as linker between the peptide and the fluorophore. Each coupling was carried out using 4 equiv of HATU, 4 equiv of 6-(Fmoc-amino) caproic acid (70.2 mg, 0.2 mmol) and 6 equiv DIEA 0.2 M in DMF for 30 min. Finally, 5(6)-carboxytetramethylrhodamine was coupling to the free amino group of the last unit of the caprionic acid using 3 equiv of the rhodamine (0.15 mmol, 64.5 mg), 3 equiv of HATU and 5 equiv of DIEA 0.2 M in DMF for 30 min. The deprotection step were carried out following standard conditions described before, the resulting crude mixtures were purified by RP-HPLC obtaining the desired peptides on yields between 25-30%.



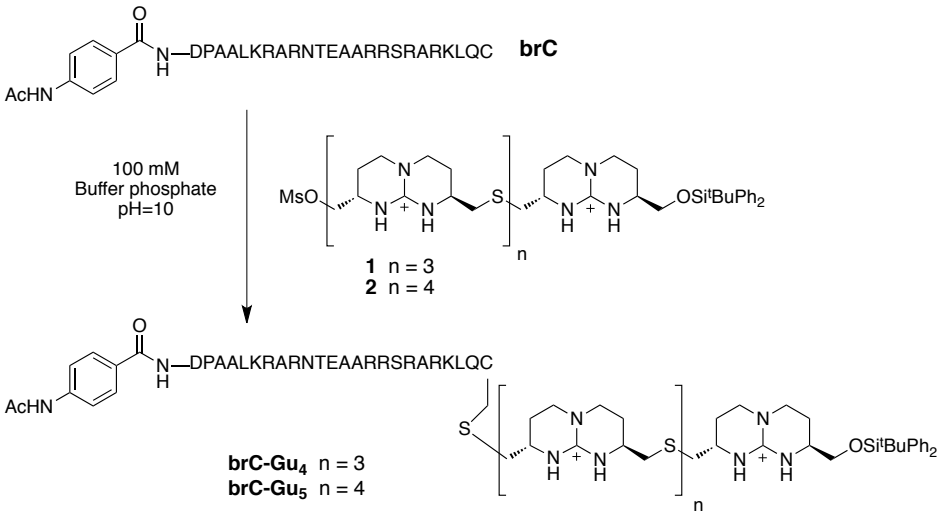
Synthesis of Br-CH_2-CO-**GGR_8**

The peptide was synthesized by the previously described SPPS procedures using 121 mg of peptide-resin (approx 23.1 μmol). Once removed the Fmoc group of the last amino acid, the peptide was stirred for 30 min at rt with a solution of bromoacetic anhydride[4] (60 mg., 231 μmol) in DCM (720 μl). Then, the resin was washed with DMF (2 × 5 mL, 2 min) and filtered. The deprotection step were carried out following standard conditions described before, the resulting crude mixtures were purified by RP-HPLC obtaining the desired peptide on yields between 35-40%.



Synthesis of **brC-Gu_4** ***and*** **brC-Gu_5**

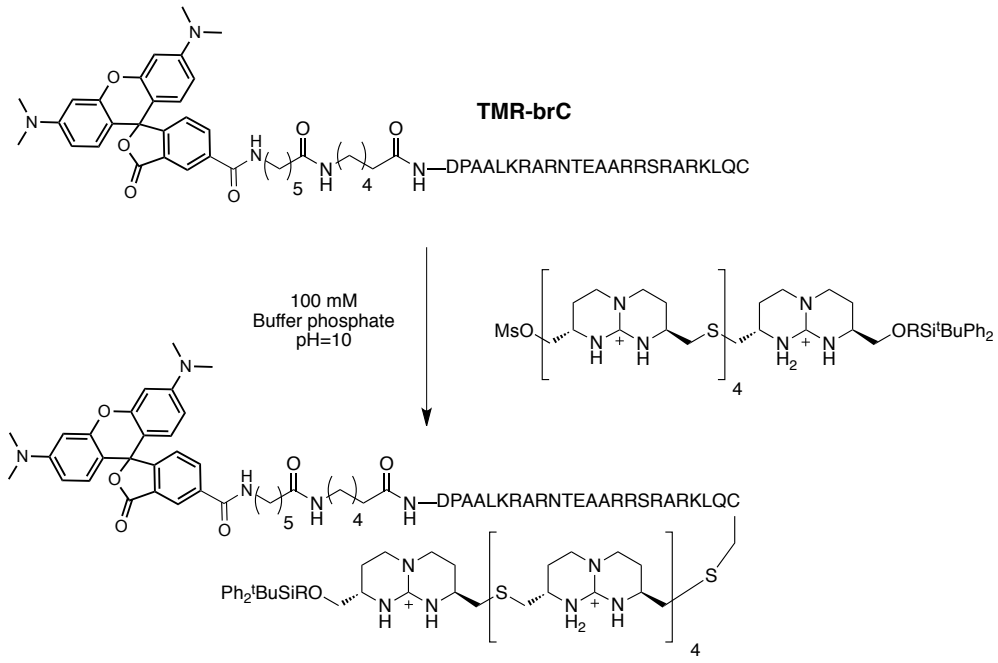
Peptide **brC** (2.28 mg, 0.6 μmol) was dissolved in a deoxygenated 100 mM buffer phosphate, pH 10 (600 μl). **1** (3 mg, 1.8 μmol) or **2** (3.5 mg, 1.8 μmol) were dissolved in a deoxygenated CH_3CN ([**1**] or [**2**] = 3 mM) and this solution was added to the peptide solution. The mixture was stirred for 14 h at 40 ºC. The reaction was quenched with 0.1% aqueous TFA, and the resulting crude purified by RP-HPLC. The collected fractions were lyophilized and stored at –20 °C, and the final products confirmed by MALDI-TOF mass spectrometry. The different conjugates were obtained as white powders with a yield of 45% for **brC-Gu_4** and 28% for **brC-Gu_5**.



4 M. Ma, A. Paredes and D. Bong, *J. Am. Chem. Soc.* **2008**, *130*, 14456.

***Synthesis of* TMR-brC-Gu$_5$**

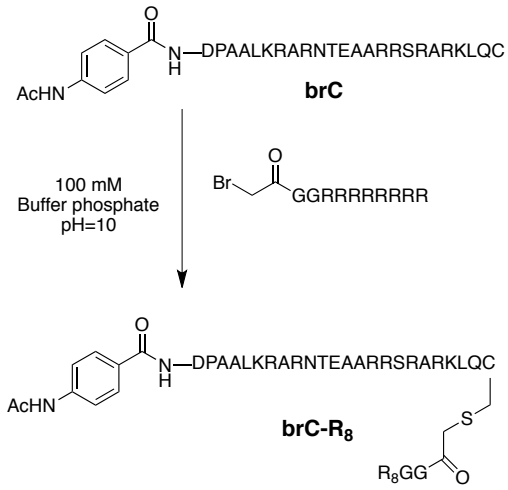
The peptide **TMR-brC** (1.3 mg, 0.3 μmol) was dissolved in a deoxygenated 100 mM buffer phosphate, pH 10 ([**TMR-brC**] = 1 mM). **2** (1.7 mg, 0.9 μmol) was dissolved in a deoxygenated CH_3CN ([**2**] = 3 mM) and this solution was added to the peptide solution. The mixture was stirred for 14 h at 40 ºC. The reaction was quenched with 0.1% aqueous TFA, and the resulting crude purified by RP-HPLC. The collected fractions were lyophilized and stored at –20 °C, and the final products confirmed by MALDI-TOF mass spectrometry. The conjugate was obtained as pink powders on yields between 25-20% for **TMR-brC-Gu$_5$**.



TMR-brC-Gu$_5$

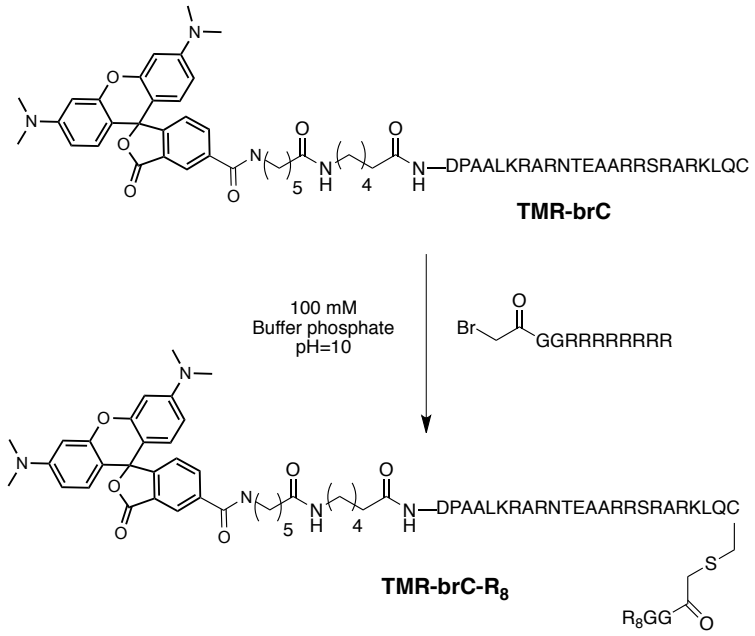
***Synthesis of* brC-R$_8$**

The peptide **brC** (2.28 mg, 0.6 μmol) was mixed with Br-CH_2-CO-**GGR$_8$** (1.44 mg, 0.6 μmol) in a deoxygenated 100 mM buffer phosphate, pH 7.5 ([Br-CH_2-CO-**GGR$_8$**] = 6 mM). The mixture was stirred for 1 h at rt. The reaction was quenched with 0.1% aqueous TFA, and the resulting crude purified by RP-HPLC. The collected fractions were lyophilized and stored at –20 °C, and the final products confirmed by MALDI-TOF mass spectrometry. The different conjugates were obtained as white powders on yields between 50-40%.



***Synthesis of* TMR-brC-R_8**

The peptide**TMR-brC** (2.64 mg, 0.6 μmol) was mixed with Br-CH_2-CO-**GGR_8** (1.44 mg, 0.6 μmol) were dissolved in a deoxygenated 100 mM buffer phosphate, pH 7.5 ([Br-CH_2-CO-**GGR_8**] = 6 mM). The mixture was stirred for 1 h at rt. The reaction was quenched with 0.1% aqueous TFA, and the resulting crude purified by RP-HPLC. The collected fractions were lyophilized and stored at –20 °C, and the final products confirmed by MALDI-TOF mass spectrometry. The different conjugates were obtained as pink powders on 30% of yield.



Analytical Data

brC

ESI-MS $[MH]^+$ calcd. for $C_{120}H_{209}N_{48}O_{34}S$ = 2900.3, found = 2899.3. UV (H_20) λ_{max}, nm (ε): 270 (18,000 $M^{-1}cm^{-1}$).

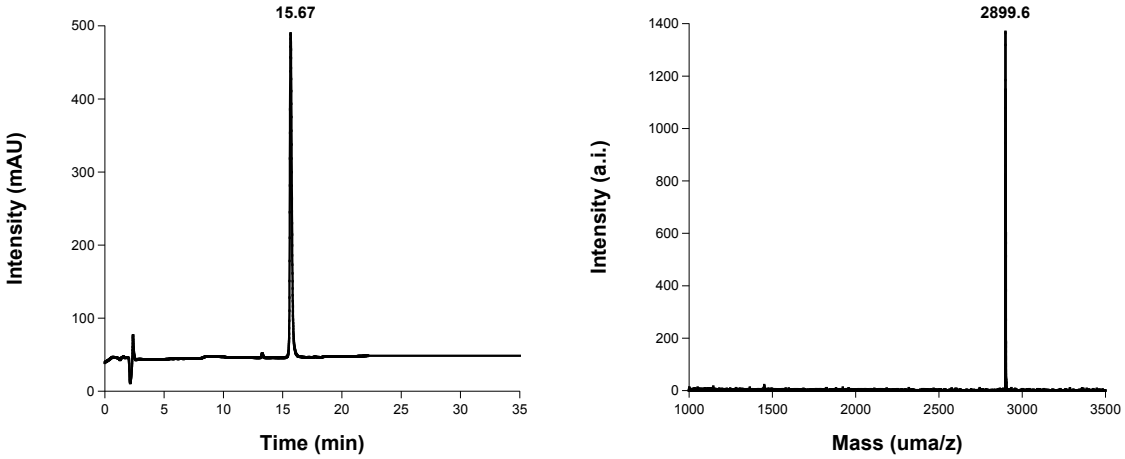


Figure 1. Left: HPLC chromatogram of the purified **brC** at 222 nm (gradient: isocratic 5% for 5 min, 5% → 75% for 30 min acetonitrile/water, 0.1% TFA; flow: 1 mL/min). Right: MALDI of the peptide **brC**.

Br-CH_2-CO-**GGR_8**

ESI-MS $[MH]^+$ calcd. for $C_{54}H_{107}BrN_{35}O_{11}$ = 1502.55, found = 1501.90

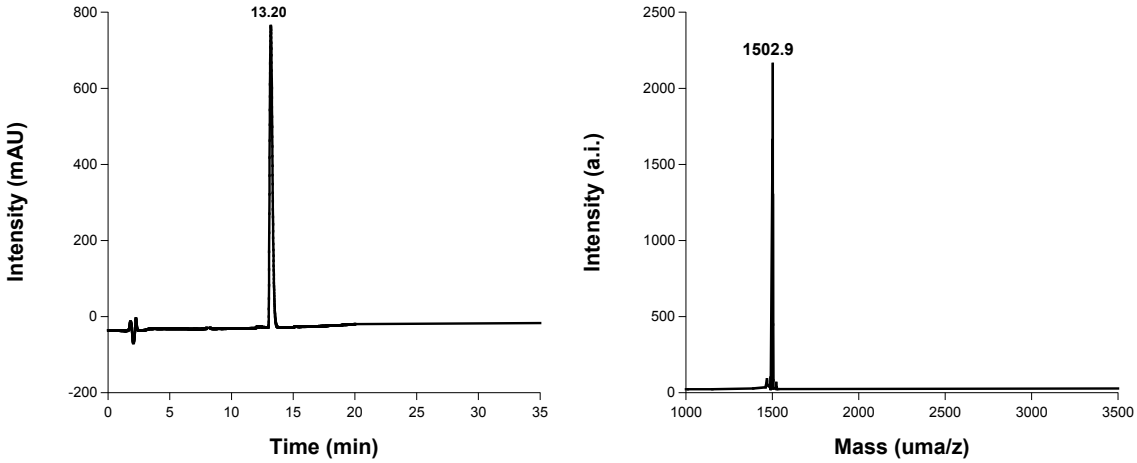


Figure 2. Left: HPLC chromatogram of the purified **Br-CH2-CO-GGR_8** at 222 nm (gradient: isocratic 5% for 5 min, 5% → 75% for 30 min acetonitrile/water, 0.1% TFA; flow: 1 mL/min). Right: MALDI of the peptide **Br-CH2-CO-GGR_8**.

TMR-brC

ESI-MS $[MH]^+$ calcd. for $C_{148}H_{244}N_{51}O_{38}S$ = 3377.9, found = 3376.7. UV (H_20) λ_{max}, nm (ε): 556 (89,000 $M^{-1}cm^{-1}$).

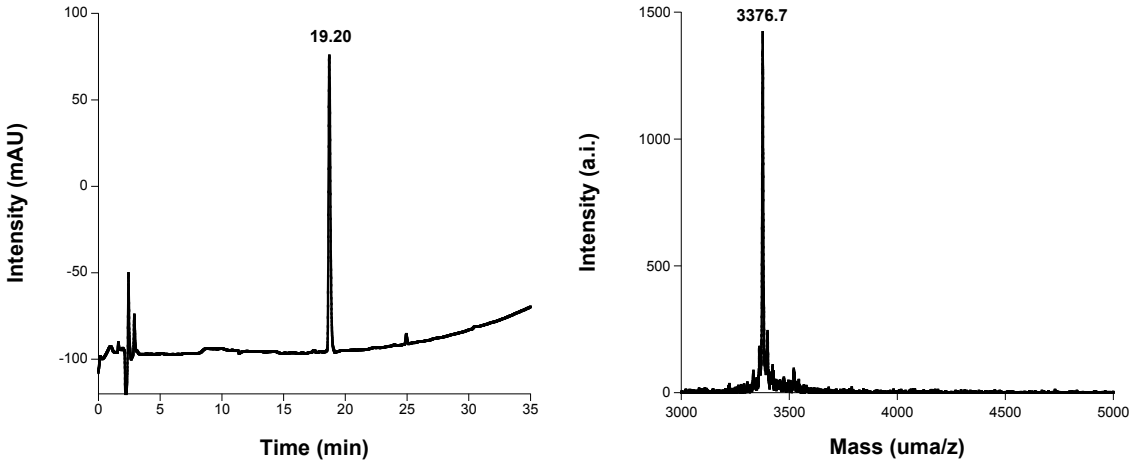


Figure 3. Left: HPLC chromatogram of the purified **TMR-brC** at 222 nm (gradient: isocratic 5% for 5 min, 5% → 75% for 30 min acetonitrile/water, 0.1% TFA; flow: 1 mL/min). Right: MALDI of the peptide **TMR-brC**.

brC-Gu_4

ESI-MS $[MH]^+$ calcd. for $C_{172}H_{287}N_{60}O_{35}S_4Si$ = 3911.8, found = 3911.0. UV (H_20) λ_{max}, nm (ε): 270 (18,000 $M^{-1}cm^{-1}$).

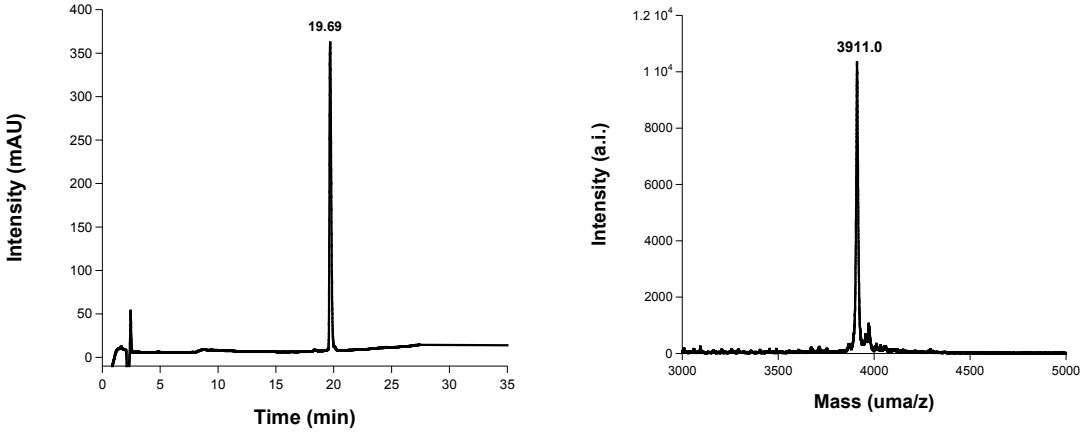


Figure 4. Left: HPLC chromatogram of the purified conjugate **brC-Gu_4** at 222 nm (gradient: isocratic 5% for 5 min, 5% → 75% for 30 min acetonitrile/water, 0.1% TFA; flow: 1 mL/min). Right: MALDI of the peptide **brC-Gu_4**.

brC-Gu$_5$

ESI-MS $[MH]^+$ calcd. for $C_{181}H_{302}N_{63}O_{35}S_5Si$= 4109.1, found = 4108.1. UV (H_20) λ_{max}, nm (ε): 270 (18,000 $M^{-1}cm^{-1}$).

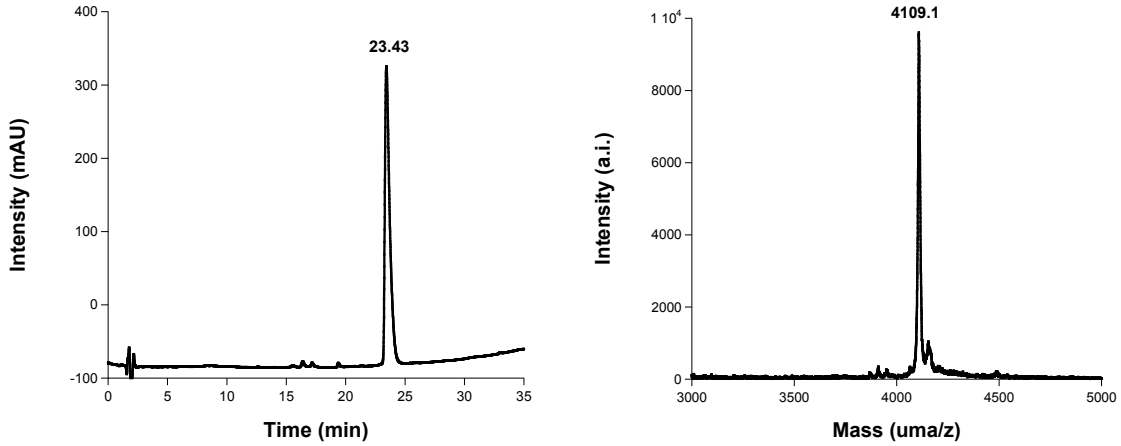


Figure 5. Left: HPLC chromatogram of the purified **brC-Gu$_5$** at 222 nm (gradient: isocratic 5% for 5 min, 5% → 75% for 30 min acetonitrile/water, 0.1% TFA; flow: 1 mL/min). Right: MALDI of the peptide **brC-Gu$_5$**.

TMR-brC-Gu$_5$

ESI-MS $[MH]^+$ calcd. for $C_{209}H_{337}N_{66}O_{39}S_5Si$= 4587.7, found = 4587.1. UV (H_20) λ_{max}, nm (ε): 556 (89,000 $M^{-1}cm^{-1}$).

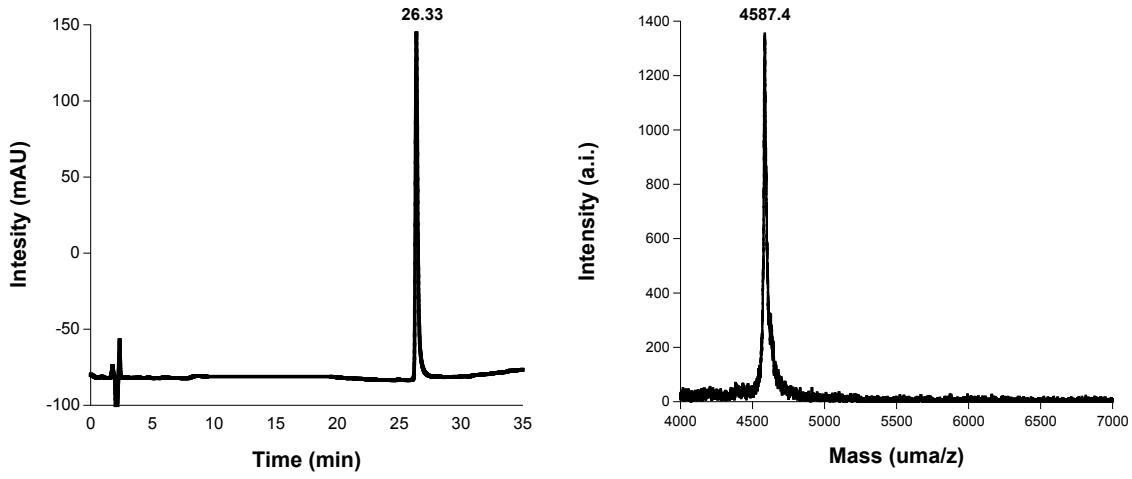


Figure 5. Left: HPLC chromatogram of the purified **TMR-brC-Gu$_5$** at 222 nm (gradient: isocratic 5% for 5 min, 5% → 75% for 30 min acetonitrile/water, 0.1% TFA; flow: 1 mL/min). Right: MALDI of the peptide **TMR-brC-Gu$_5$**.

brC-R_8

ESI-MS $[MH]^+$ calcd. for $C_{147}H_{313}N_{82}O_{46}S$ = 4321.9, found = 4321.0.

UV (H_20) λ_{max}, nm (ε): 270 (18,000 $M^{-1}cm^{-1}$).

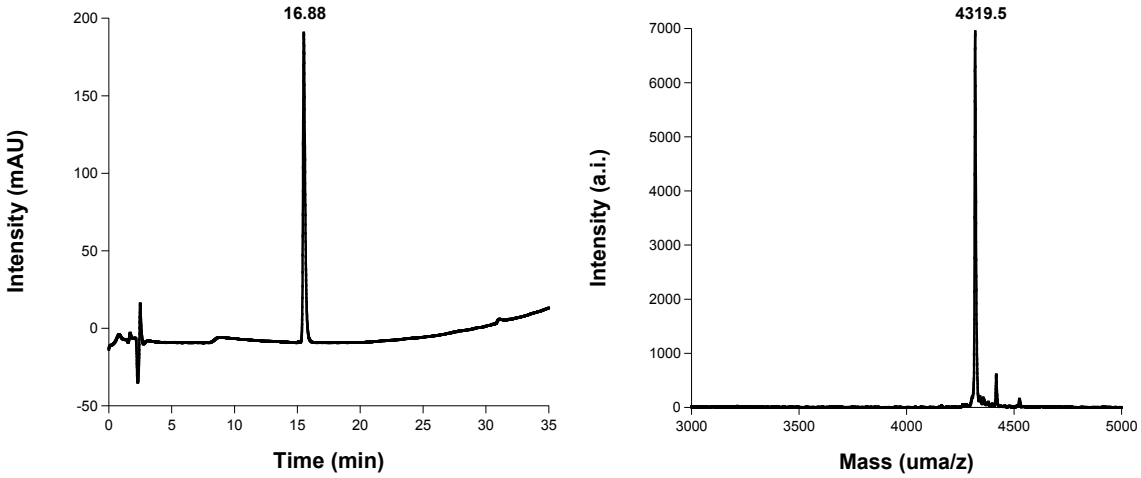


Figure 7. Left: HPLC chromatogram of the purified **brC-R_8** at 222 nm (gradient: isocratic 5% for 5 min, 5% → 75% for 30 min acetonitrile/water, 0.1% TFA; flow: 1 mL/min). Right: MALDI of the peptide **brC-R_8**.

TRM-brC-R_8

ESI-MS $[MH]^+$ calcd. for $C_{202}H_{348}N_{85}O_{50}S$ = 4797.7, found = 4797.2.) λ_{max}, nm (ε): 556 (89,000 $M^{-1}cm^{-1}$).

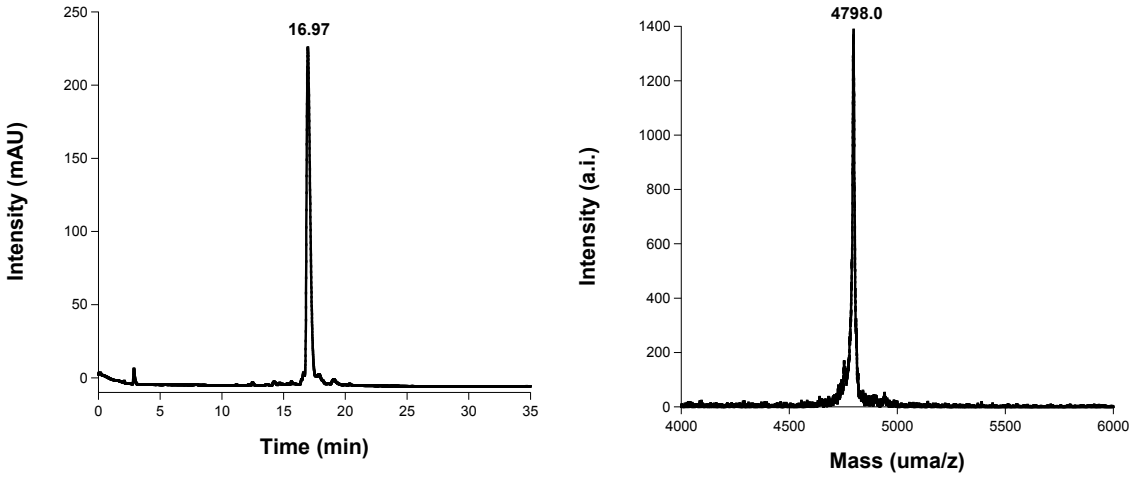
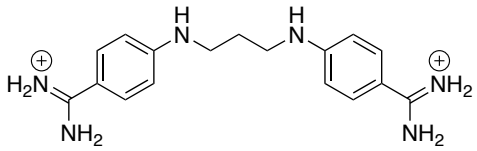


Figure 7. Left: HPLC chromatogram of the purified **TMR-brC-R_8** at 222 nm (gradient: isocratic 5% for 5 min, 5% → 75% for 30 min acetonitrile/water, 0.1% TFA; flow: 1 mL/min). Right: MALDI of the peptide **TMR-brC-R_8**.

Fluorescence Spectroscopy

Competition assays of **3** with the minor groove binders **S1** and the dsDNA **A/T**.



S1

Figure 9: S1 interacts with the sequence AATT in a dsDNA with a *Kd*= 80 nM at 30-100 mM of NaCl and 281 nM in the EMSA conditions.[5]

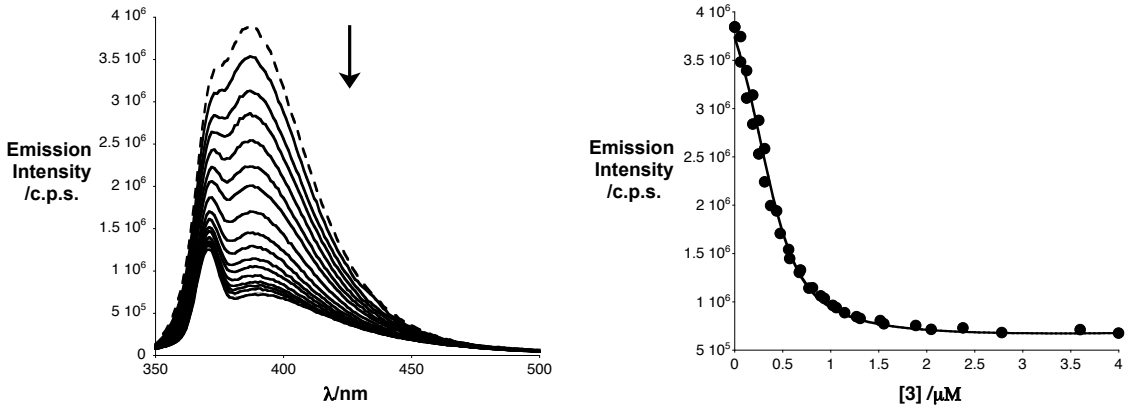


Figure 10: *Left:* Dash line: fluorescence emission spectrum of 0.5 μM solution of **S1** in Tris-HCl buffer 7 mM pH 7.5, **30 mM NaCl**, with 0.7 μM of dsDNA **A/T**. Solid lines: fluorescence emission spectra of S**1** after additions of increasing amounts of the pentaguanidinium **3**. *Righ*t: Displacement curve. The estimated *Kd* value is the average of two experiments: *Kd*= 9 ± 1 nM. Emission monitored at 390 nm. The curve represents the best fit to the data using nonlinear analysis with the DynaFit program (Biokin Software)[6] to the equation derived for a 1:1 model of both molecules. Kd of **S1** in this conditions is 80 nM).

5 O. Vázquez, M. I. Sánchez, J. Martínez Costas, E. Vázquez Sentís, J. L. Mascareña *Org. Lett* **2010**, *12*, 216.
6 P. Kuzmic *Anal. Biochem.* **1996**, *237*, 260.

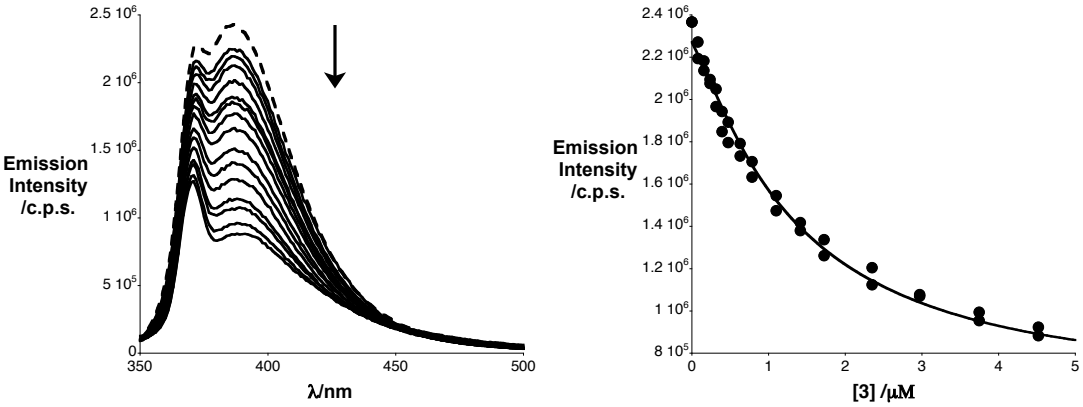


Figure 11: *Left:* Dash line: fluorescence emission spectrum of 0.5 µM solution of **S1** in Tris-HCl buffer 20 mM pH 7.5, **100 mM NaCl**, with 0.7 µM of dsDNA **A/T**. Solid lines: Solid lines: fluorescence emission spectra of S**1** after additions of increasing amounts of the pentaguanidinium **3**. *Right:* Displacement curve. The estimated *Kd* value is the average of two experiments: Kd = 144 ± 6 nM. Emission monitored at 390 nm. The curve represents the best fit to the data using nonlinear analysis with the DynaFit program (Biokin Software)[6] to the equation derived for a 1:1 model of both molecules (Kd of **S1** in this conditions is 80 nM).

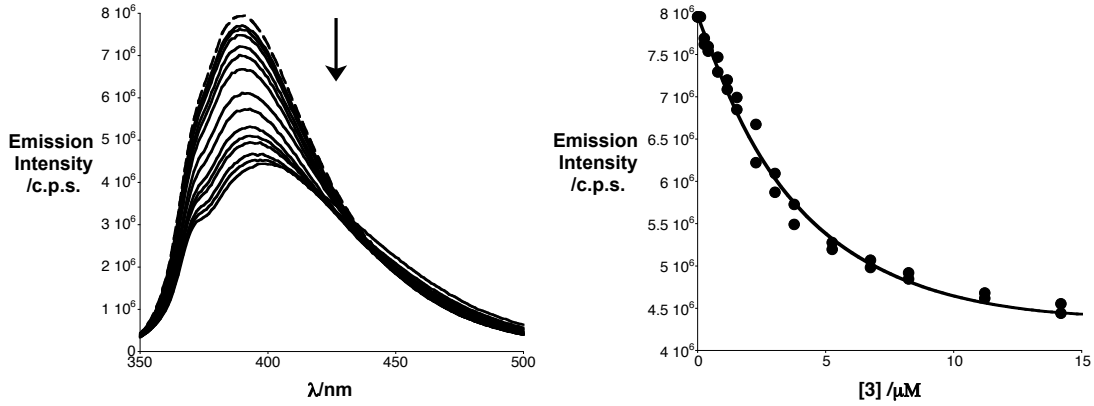


Figure 12: *Left:* Dash line: fluorescence emission spectrum of 1 µM solution of **S1,** Using 1 µM of **S1**, 1.5 µM of dsDNA **A/T**, **90 mM KCl, 1.8 mM $MgCl_2$, 3.6 mM AEDT, 2.2% of BSA** and Tris-HCl buffer 20 mM pH 7.5. Solid lines: fluorescence emission spectra of **S1** after additions of increasing amounts of the pentaguanidinium **3**. *Right:* Displacement curve. The estimated *Kd* value is the average of two experiments: Kd = 782 ± 8 nM. Emission monitored at 390 nm. The curve represents the best fit to the data using nonlinear analysis with the DynaFit program (Biokin Software)[6] to the equation derived for a 1:1 model of both molecules (Kd of **S1** in this conditions is 281 nM).

Circular Dichroism (CD)

Measurements were made with a *Jasco-715* coupled with a thermostat *Nestlab RTE-111*. The settings used were: Acquisition range: 320-195nm; band width: 2.0 nm; resolution: 0.2 nm; accumulation: 3 scans; sensitivity 10 mdeg; response time: 0.25 s, speed: 100 nm/min. CD measurements were made in a 2 mm cell at 20 ºC.

Circular dichroism for conjugates:

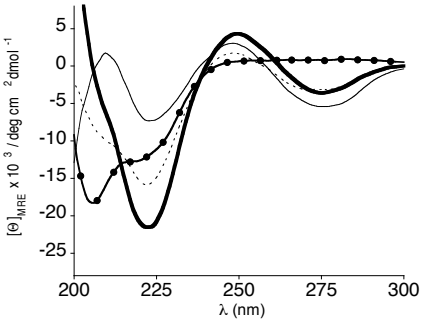


Figure 13: Circular dichroism of a 5.0 µM solution of **brC-Gu$_5$** in the absence of DNA (●), in the presence of 1 equiv of **AP1^{hs}•A/T** (bold solid line), in the presence of 1 equiv of **AP1^{hs}•mA/T** (dash line), and in the presence of 1 equiv of **mAP1^{hs}•A/T** (solid line). The samples were dissolved in 10 mM phosphate buffer (pH 7.5) and 100 mM of NaCl at 20 ºC. The contribution of the parent DNA to the CD spectrum of the complexes has been subtracted.

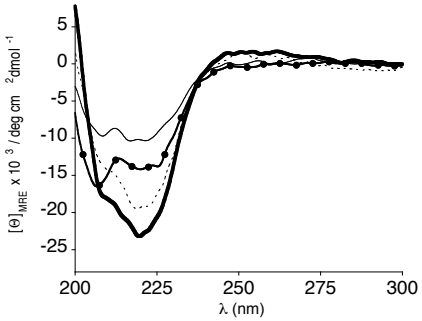


Figure 14: Circular dichroism of a 5.0 µM solution of **brC-R$_8$** in the absence of DNA (●), in the presence of 1 equiv of **AP1^{hs}•A/T** (bold solid line), and in the presence of 1 equiv of **AP1^{hs}•mA/T** (dash line), in the presence of 1 equiv of **mAP1^{hs}•A/T** (solid line). The samples were dissolved in 10 mM phosphate buffer (pH 7.5) and 100 mM of NaCl at 20 ºC. The contribution of the parent DNA to the CD spectrum of the complexes has been subtracted.

Circular dichroism experiments with pentaguanidinium **3**:

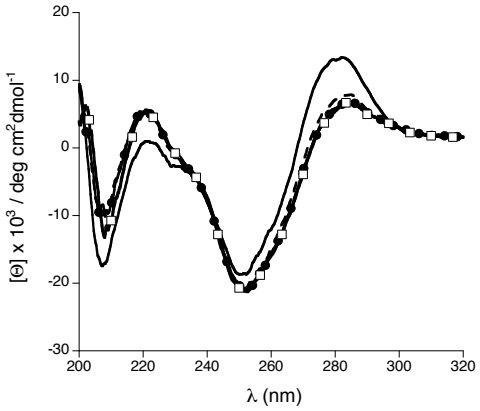


Figure 15: Circular dichroism spectra of a 25 µM solution of dsDNA **A/T** (solid line) in phosphate buffer 10 mM; 100 mM NaCl, pH 7.5; same solution after addition of 1 equiv (dash line), 2 equiv (●), 3 equiv (□) of **3**.

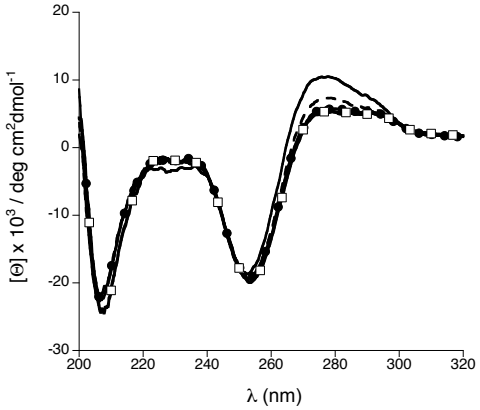


Figure 16: Circular dichroism spectra of a 25 µM solution of dsDNA **mA/T** (solid line) in phosphate buffer 10 mM; 100 mM NaCl, pH 7.5; same solution after addition of 1 equiv (dash line), 2 equiv (●), 3 equiv (□) of **3**.

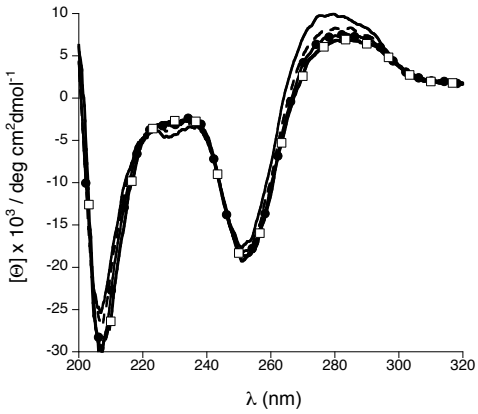


Figure 17: Circular dichroism spectra of a 25 μM solution of dsDNA **G/C** (solid line) in phosphate buffer 10 mM; 100 mM NaCl, pH 7.5; same solution after addition of 1 equiv (dash line), 2 equiv (●), 3 equiv (□) of **3**.

DNA labeling with ^{32}P

The labeling with ^{32}P was accomplished in the following manner: 1 μL of the single strand oligonucleotide which will be labeled (10 μM), 1 μL kinase buffer 10x (40 mM, Tris HCl pH 7.5, 10 mM $MgCl_2$, 5 mM DTT), 2 μL ATP* (32 ϒ-ATP [5000 Ci/mmol]) and 0.5 μL Ty kinase (10 units / μL); after shaking mixture is heated for 1 h at 37ºC, the is quenched by heating for 10 min at 95-100 ºC.

DNA annealing takes place when another solution with complementary single strand oligonucleotide (2 μL DNA (10 μM), 1 μL kinase buffer 10x, 7 μL H_2O) is added to the above mixture at rt, the mixture is heated for 10 min at 90ºC and left to reach rt. Finally 2 μL of this stock is diluted to 198 μL of H_2O.

Electrophoretic Mobility Shift Assay

EMSA was performed with a *BIO-RAS Mini Protean* gel system, powered by an electrophoresis power supplies *PowerPac Basic* model, maximum power 150 V, frequency 50.60 Hz at 140 V (constant V).

For radioactive EMSA: Incubation binding mixtures (20 µL) contained 18 mM Tris HCl (pH 7.5), 50 mM KCl, 1.2 mM Mg_2Cl, 0.5 mM EDTA, 9% glycerol, 0.11 mg/mL BSA and 4.2 % NP-40 and 45 pM of the desired radioactive ds-oligonucleotide and 50 nM of the same oligonucleotide without radioactive labeling. After incubation for 45 min the samples were loaded and run into a 10% nondenaturing polyacrylamide gel using TBE 0.5x buffer (44.5 mM Tris, 44.5 mM Boric acid, 1mM EDTA pH 8.0) for 45 min at rt.

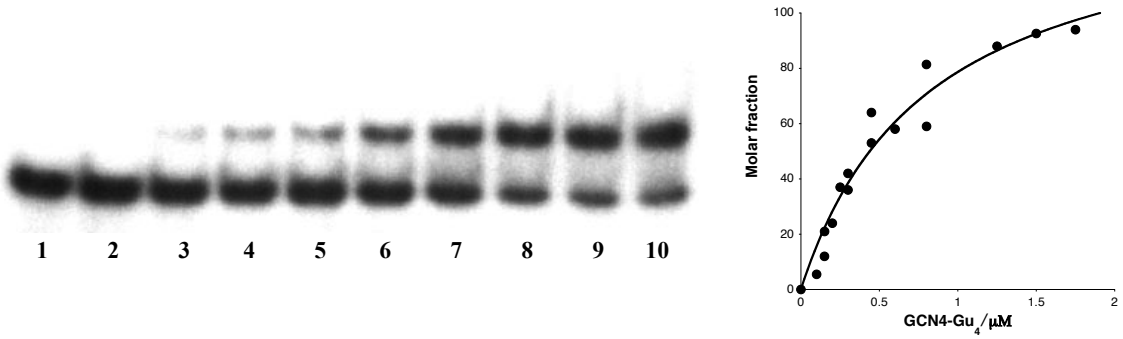


Figure 18: EMSA results showing the binding of **brC-Gu$_4$** to dsDNA **AP1^{hs}•A/T** [50 nM]. Lane 1-10: **AP1^{hs}•A/T** + [**brC-Gu$_4$**] = 0, 50, 100, 150, 200, 250, 300, 450, 600, 800 nM. The curve represents the best fit to the data using nonlinear analysis with the DynaFit program (Biokin Software)[6] to the equation derived for a 1:1 model of both molecules (Kd is 813 ± 170 nM).

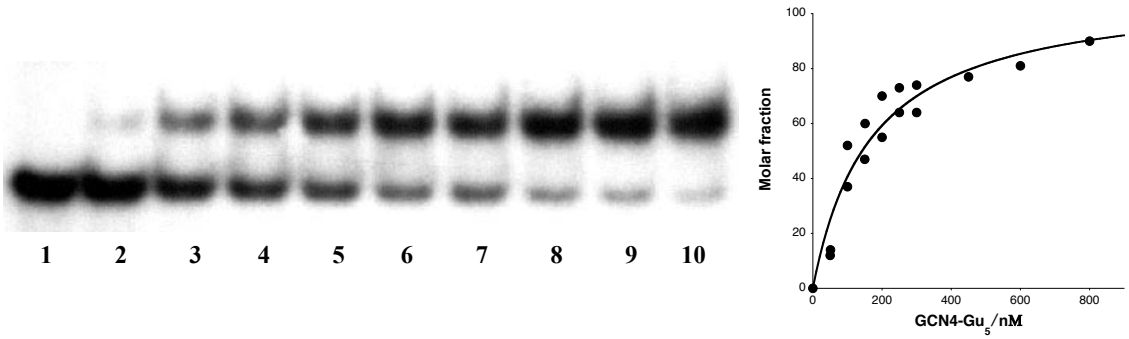


Figure 19: EMSA results showing the binding of the **brC-Gu$_5$** to dsDNA **AP1^{hs}•A/T** [50 nM]. Lane 1-10: **AP1^{hs}•A/T** + [**brC-Gu$_5$**] = 0, 50, 100, 150, 200, 250, 300, 450, 600, 800 nM. The curve represents the best fit to the data using nonlinear analysis with the DynaFit program (Biokin Software)[6] to the equation derived for a 1:1 model of both molecules (Kd is 169 ± 37 nM).

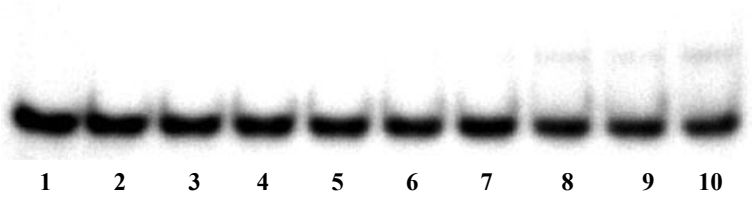


Figure 20: EMSA results showing the binding of the **brC-Gu$_4$** to dsDNA **AP1^{hs}•mA/T** [50 nM]. Lane 1-10: **AP1^{hs}•mA/T** + [**brC-Gu$_4$**] = 0, 50, 100, 150, 200, 250,300,450, 600, 800 nM.

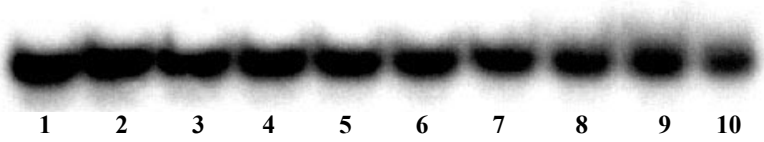


Figure 21: EMSA results showing the binding of the **brC-Gu$_4$** to dsDNA **mAP1^{hs}•A/T** [50 nM]. Lane 1-10: **mAP1^{hs}•A/T** + [**brC-Gu$_4$**] = 0, 100, 150, 20, 250, 300, 450, 600, 800, 1000 nM.

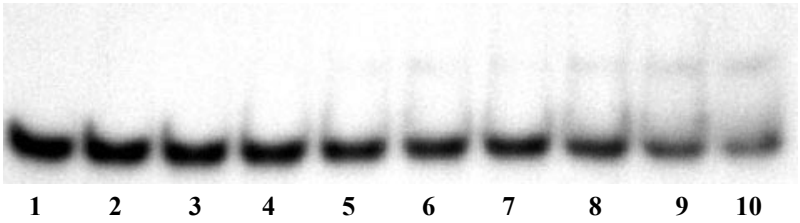


Figure 22: EMSA results showing the binding of the **brC-Gu$_5$** to dsDNA **AP1^{hs}•mA/T** [50 nM]. Lane 1-10: **AP1^{hs}•mA/T** + [**brC-Gu$_5$**] = 0, 50, 100, 150, 200, 250,300,450, 600, 800 nM.

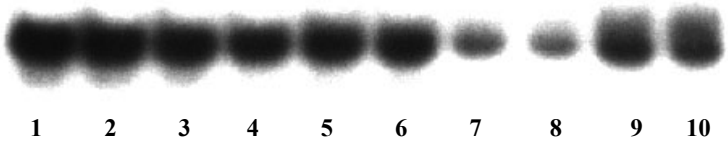


Figure 23: EMSA results showing the binding of the **brC-Gu$_5$** to dsDNA **mAP1^{hs}•A/T** [50 nM]. Lane 1-10: **mAP1^{hs}•A/T** + [**brC-Gu$_5$**] = 0, 100, 150, 20, 250, 300, 450, 600, 800, 1000 nM.

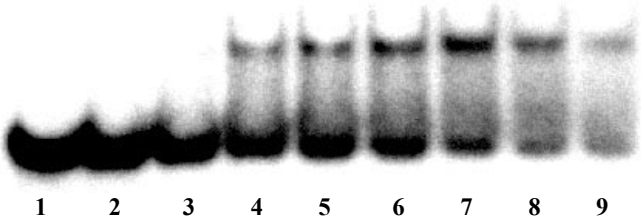


Figure 24: EMSA results showing the binding of the **brC-R$_8$** to dsDNA **AP1^{hs}•A/T** [50 nM]. Lane 1-10: **AP1^{hs}•A/T** + [**brC-R$_8$**] = 0, 50, 100, 150, 200, 250, 300, 450, 600, 800 nM.

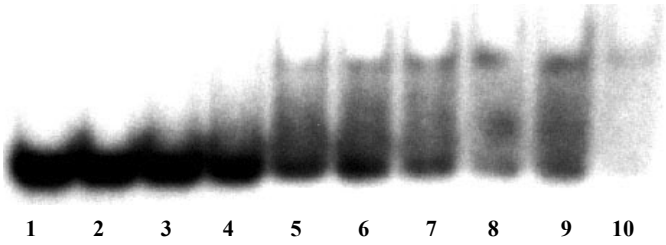


Figure 25: EMSA results showing the binding of the **brC-R$_8$** to dsDNA **AP1^{hs}•mA/T** [50 nM]. Lane 1-10: **AP1^{hs}•mA/T** + [**brC-R$_8$**] = 0, 50, 100, 150, 200, 250, 300, 450, 600, 800 nM.

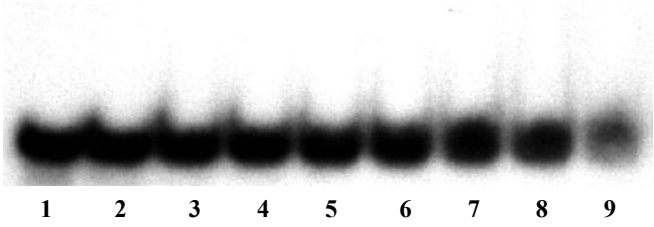


Figure 26: EMSA results showing the binding of the **brC-R$_8$** to dsDNA **mAP1^{hs}•A/T** [50 nM]. Lane 1-10: **mAP1^{hs}•A/T** + [**brC-R$_8$**] = 0, 50, 100, 150, 200, 250, 300, 450, 600, 800 nM.

Non-quantitative EMSA assays using *SyBrGold* staining: Binding reactions were performed over 30 min in 18 mM Tris HCl (pH 7.5), 90 mM KCl, 1.8 mM Mg_2Cl, 1.8 mM EDTA, 9% glycerol, 0.11 mg/mL BSA and 2.2 % NP-40. In the experiments we used 50 nM of the unlabeled dsDNAs. After incubation for 30 min. products were resolved by PAGE using a 10% non-denaturing polyacrylamide gel and 0.5X TBE buffer for 40 min at rt, and analyzed by staining with *SyBrGold* (Molecular Probes: 5 µL in 50 mL of TBE) for 10 min. and visualized by fluorescence. 5X TBE buffer: 0.445M Tris HCl, 0.445 M Boric acid, 10 mM ETDA pH 8.0.

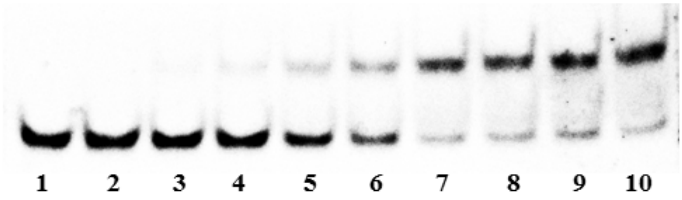


Figure 27: EMSA analysis (SybrGold staining) of the DNA selectivity showing the binding of the **brC-Gu$_5$** to dsDNA **AP1^{hs}·A/T** [50 nM]. Lane 1-10: **AP1^{hs}·A/T** + [**brC-Gu$_5$**] = 0, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.25, 1.5 µM.

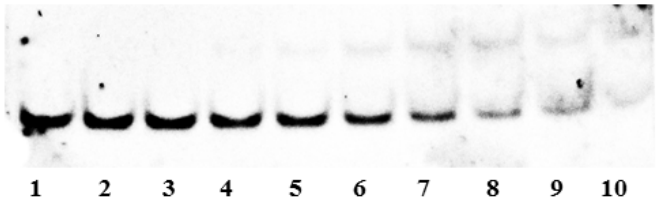


Figure 28: EMSA analysis (SybrGold stainnning) of the DNA selectivity showing the binding of the **brC-Gu$_5$** to dsDNA **AP1^{hs}·G·A/T** [50 nM]. Lane 1-10: **AP1^{hs}·G·A/T** + [**brC-Gu$_5$**] = 0, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.25, 1.5 µM.

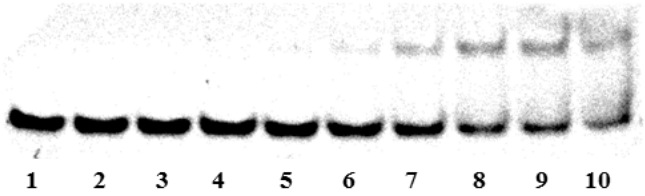


Figure 29: EMSA analysis (SybrGold staining) of the DNA selectivity showing the binding of the **brC-Gu$_5$** to dsDNA **AP1^{hs}·GGG·A/T** [50 nM]. Lane 1-10: **AP1^{hs}·GGG·A/T** + [**brC-Gu$_5$**] = 0, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.25, 1.5 µM.

Cell culture experiments

Vero cells were maintained in DMEM (Dulbecco Modified Eagle Medium) containing 10% of FBS (Fetal Bovine Serum). The day before the cellular uptake experiments, cells were seeded in twelve well plates containing glass coverslips (15 mm). Cells were then washed 3 times with PBS and overlaid with 1 mL of fresh DMEM, and no serum is added. Samples with the indicated concentration of the rhodamine-labeled conjugates were added, and the mixtures incubated for 30 min in an incubator at 37ºC; then the medium is removed and the cells are washed with PBS (3 × 1 mL) prior visualization.

Co-staining experiments were performed to clarify the intracellular distribution of the rhodamine-labeled conjugates, using DAPI as a bone fide DNA fluorescent probe.

Images were obtained with an Olympus DP-71 digital camera mounted on an Olympus BX51 fluorescence microscope equipped with a built-in Koehler illumination for transmitted light 12 V 100 W halogen bulb Light, and a U-RFL-T power supply unit housing a USH102D 20 V, 100 Watt mercury arc lamp. Images were further processed (cropping, resizing and contrast global contrast and brightness adjustment) with Adobe Photoshop (Adobe Systems). All images were taken at ISO 400 sensitivity. The parameters of the fluorescent channels are the following: Blue channel: Ultraviolet excitation U-MWU2: excitation filter 360-370 nm, emission filter 420 nm and dichromatic mirror 400 nm; Red channel: Green excitation U-MNG2: excitation filter 530-550 nm, emission filter 590 nm and dichromatic mirror 570 nm.

Trypan Blue assay: Toxicity was evaluated after incubation with 5 μM **brC-Gu$_5$** for 30 min, then the cells were washed 3 times with PBS, overlaid with 1 mL of Trypan Blue 2.0% in PBS 1X for 2.5 min and visualized under the microscope. Dead cells appear as dark blue spots (pointed with arrows).

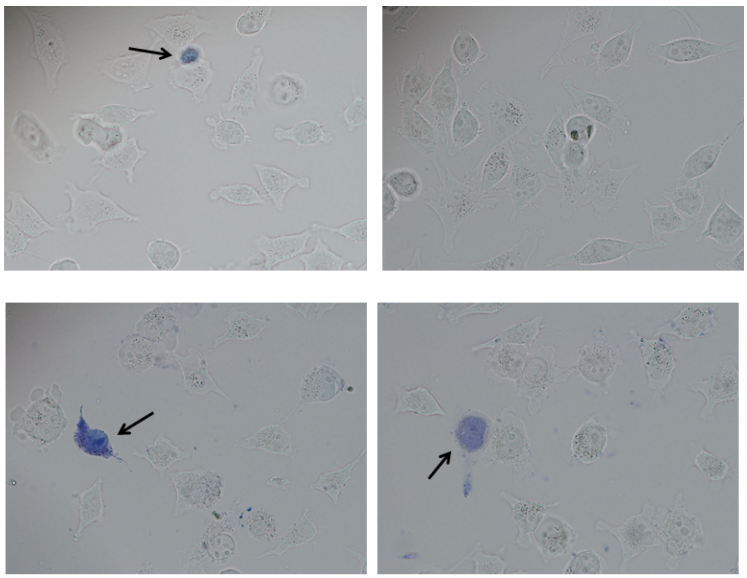


Figure 30: Top: Vero cells incubated with Trypan Blue; bottom: Vero cells incubated with Trypan Blue after being treated with **brC-Gu$_5$** 5 μM for 30 min. Pictures were taken at 400X. The black arrows indicates dead cells that show as intense blue.