

High Affinity, Sequence Specific DNA Binding by Synthetic Tripyrrole–Peptide Conjugates

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Abstract: Linking the basic region of a bZIP transcription factor to a distamycin-like tripyrrole peptide by means of a nitrogen-containing tether produces a hybrid capable of high-affinity recognition of specific, designated DNA sequences. The importance of the nitrogen in the tether is shown by the considerable reduction in affinity (more

than 10-fold) caused by its replacement with an ether linkage. Attachment of an aminopropyl chain on the pyrrole adjacent to the pyrrole bearing the ni-

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trogen-containing tether increases affinity approximately one order of magnitude. These results confirm that a suitable location of protonated amine groups on designed DNA-binding peptides provides for higher affinities, most probably because of the generation of salt bridged contacts with the phosphodiester backbone.

Introduction

The proper functioning of cells involves accurate control of DNA transcription by the concerted and regulated action of transcription factors. These proteins interact, with high affinity and specificity, with short DNA sequences that are usually located upstream of the promoter of the gene.^[1] The design of molecules that are smaller than natural transcription factors but have similar DNA-binding properties is an important goal of current research at the chemistry/biology interface, not only because of their potential utility for elucidation of the molecular basis of DNA recognition by natural proteins, but also as potential chemotherapeutic agents in the postgenomic age.^[2]

One of the simplest structural families of transcription factor proteins is the bZIP family, which bind dsDNA as leucine zipper-mediated homo- or heterodimers, and insert two N-terminal peptidic chains called *basic regions* (BR) into adjacent DNA major grooves.^[3] Interestingly, the basic regions are largely unstructured in the absence of DNA, but they fold into an α -helix upon specific DNA binding.^[4] Although the leucine zipper region does not contact the DNA directly, DNA binding is strongly dependent on such dimerization, as prevention of this process through specific mutations precludes DNA recognition.^[5] It has been shown that the leucine zipper unit can be replaced by other noncovalent or by covalent artificial dimerizing units without significantly compromising the recognition capabilities of the system.^[6] However, monovalent bZIP BRs exhibit very poor DNA-binding affinities unless the important DNA-contacted residues are appropriately grafted into a preorganized α -helix.^[7]

We have recently demonstrated that appropriate tethering of a monomeric bZIP BR domain to a distamycin-like tripyrrole that binds with moderate-to-good affinity in the minor groove adjacent to the BR target site, provides for specific binding of the peptide to its cognate major groove site, binding that most probably occurs according to the hypothetical model depicted in the Figure 1.^[8] Most important, the resulting tripyrrole–peptide conjugate exhibits higher affinity for its target hybrid site than either the BR monomer or the tripyrrole. In particular we have found that the derivative **1**, which has a peptidic region of only 22 natural amino acids, is capable of binding relatively long specific DNA

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Supporting information for this article is available on the WWW under <http://www.chemeurj.org/> or from the author: Synthesis of **3**, **6**, **10**, **12**, and **15** and characterization data, as well as gel shift titration data for the binding of **11** to T/CRE^{bs} at 4°C.

sites with low nanomolar affinity ($K_d \sim 3$ nM) at 4°C. It should be noted, however, that part of this affinity is lost when the interaction assay is carried out at 23°C, a fact that somewhat restricts the potential utility of the system as a sequence-specific DNA probe.

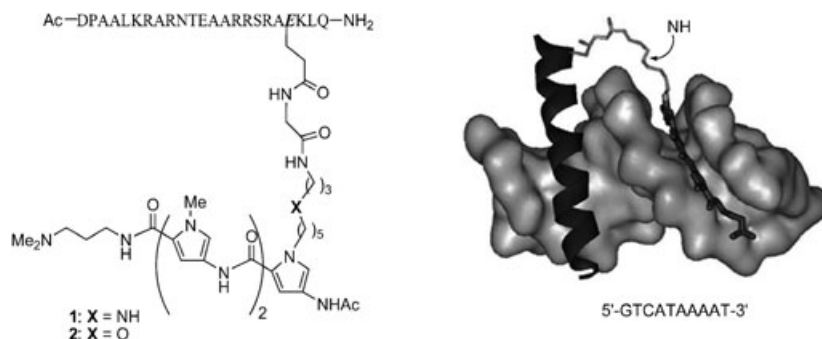


Figure 1. Structure of the peptide-tripyrrole hybrids, and qualitative working model for the hypothetical interaction of **1** with the specific DNA target, showing a possible position for the secondary NH group of the tether (Caution: the drawing doesn't necessarily reflect the real structure of the complex).

Significantly, we have found that the linking tether is not a mere connector that keeps the DNA-binding modules at an appropriate distance, but apparently it plays a more active role in the recognition process. Hence, replacement of the pentylpropylamine fragment of tether with a propyl-triglycine unit markedly reduces affinity for DNA, even though both tethers span very similar distances.^[8b] This result raised the question of whether such a decrease in affinity is due to geometric restrictions imposed by the rigidity of the peptidyl tether or to the absence of the secondary nitrogen atom present in the linker of **1**, or perhaps to both factors. Herein we demonstrate that replacing the secondary amino group present in the linker of hybrid **1** by an oxygen atom results in a considerably drop in DNA affinity. This

result suggests that such amino group, which should be mostly protonated at neutral pH, is critical for a successful DNA binding by this type of conjugates, most probably because it is engaged in a salt-bridged type of contact with the phosphodiester oxygens.

This finding supported the hypothesis that the presence of amino groups in appropriate positions of a DNA-binding ligand strengthens the interaction, and suggested that the DNA affinity of tripyrrole-peptide hybrids such as **1** might be further increased by facilitating more interactions of this kind. Indeed in this work we also show that attaching a propylamine chain to the nitrogen atom of the middle pyrrole in hybrid **1** leads to a conjugate **11** capable of binding the designated composite DNA sequence with a K_d in the low nanomolar

range even at room temperature (23°C).

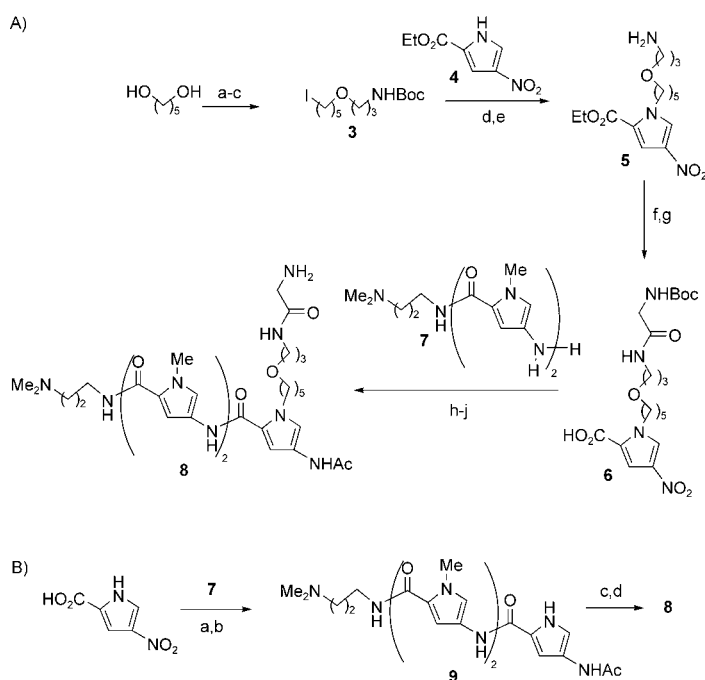
Results and Discussion

Synthesis of hybrid 2, which contains an ether type of linkage: In order to dissect the relevance of the secondary amine group of the tether in the hybrid **1** we prepared the analogue **2** which bears an oxygen atom in place of the NH group. The synthesis of **2** required assembly of aminotripyrrole **8**, which was accomplished both by linking monopyrrole **4** first to the tether and then to the N-aminodipyrrole **7**^[9] (route A in Scheme 1), and also by installing the tether directly on the N-terminal ring of tripyrrole **9** (route B).^[8b]

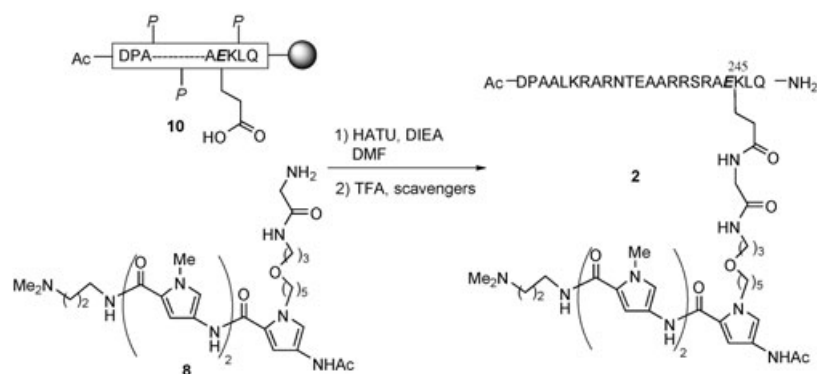
Although route A is slightly more convergent, route B is shorter, and is more versatile in that it allows the tripyrrole to be endowed with a variety of different tethers by just changing the alkylating species in the final step. With intermediate **8** in hand, we assembled compound **2** as shown in Scheme 2, by coupling **8** with a peptide consisting essentially of amino acids 226–248 of the GCN4 BR (R245 of this BR was replaced by glutamic acid for attachment of the tether).^[10] The coupling reaction was carried out while the peptide was still attached to the solid support and fully protected except at the key glutamic acid; following isolation of **2**, the overall yield of the process of peptide assembly and coupling to **8** was approximately 36%.

DNA binding properties of 2 in comparison with 1: Since specific binding of bZIP BRs to DNA is concomitant with the formation of an α -helix, circular dichroism (CD) is a particularly useful technique to detect such interactions, provided that the basic region is not already highly helical in the absence of DNA. Clearly, evidence for an increase in

Abstract in Spanish: Conectando la región básica de un factor de transcripción de tipo bZIP con un tripirrol similar a la Distamicina, a través de una cadena que contiene un grupo amino, se obtiene un híbrido capaz de reconocer secuencias específicas de ADN con gran afinidad. Si se reemplaza el grupo amino de la cadena por un éter se observa una importante reducción en la afinidad (más de 10 veces), lo que demuestra la importancia del grupo NH presente en dicha cadena. Por otra parte, la introducción de un grupo aminopropilo en el pirrol contiguo al que porta la cadena de conexión al péptido conlleva un aumento de afinidad de aproximadamente un orden de magnitud. Estos resultados confirman que una localización adecuada de grupos amino protonados en péptidos diseñados para interactuar con ADN proporciona mejores afinidades, seguramente debido a la generación de puentes salinos con los fosfodiésteres del ADN.



Scheme 1. Synthesis of the tripyrrole unit **8**: A) a) 1) NaH, THF, 23 °C; 2) acrylonitrile; b) Et₃N, Boc₂O, THF, H₂, Pd/C; c) Ph₃P, I₂, imidazole; d) **4**, K₂CO₃, acetone, reflux; e) TFA, CH₂Cl₂; f) Boc-Gly-OSu, Et₃N, CH₂Cl₂; g) NaOH, EtOH/H₂O; h) **7**, DECP, Et₃N, DMAP, THF; i) 1) H₂, Pd/C; 2) AcCl, Et₃N; j) TFA, CH₂Cl₂; B) a) DECP, Et₃N, DMAP, THF, **7**; b) 1) H₂, Pd/C; 2) AcCl, Et₃N; c) 1) **3**, K₂CO₃, acetone, reflux; 2) TFA, CH₂Cl₂; d) 1) Boc-Gly-OSu, DIEA, DMF; 2) TFA, CH₂Cl₂.



Scheme 2. Coupling step for the synthesis of **2**.

helicity by CD does not necessarily correlate with high affinity binding, as the concentrations required for the CD experiments are in the micromolar range; however, tight, specific binding does require the induction of an α -helical structure, a characteristic that can be readily detected by observation of the change in the negative ellipticity at 222 nm in the CD spectrum.^[11] As previously reported, addition of a 20 base-pair duplex oligonucleotide (T/CRE^{hs}) containing the designated target hybrid sequence (P5'-GTCATAAAAT-3') to **1** at 4 °C, produces a significant variation of the CD signal at 222 nm, which is consistent with specific binding.

This band is accompanied by increased positive ellipticity at 330 nm due to the tripyrrole moiety binding in the minor groove (Figure 2a). Addition of compound **2** to the same oligonucleotide caused parallel spectral changes to those induced by **1**, and the specificity of the interaction was supported by the failure to induce spectral changes at 222 nm when the oligonucleotide used was T/CRE^{hs}_m, which has a single mutation in the peptide-binding site, or CRE^{hs}, which lacks the A-rich sequence responsible for the minor-groove binding of the tripyrrole moiety. The intensity of the band at 222 nm in the complex **2**-T/CRE^{hs} is slightly lower than that observed in the complex with **1** but still significant, suggesting that the basic region of **2** also folds into an α -helix upon binding to the cognate composite DNA sequence.

Although this result could suggest a relatively high affinity binding, electrophoretic mobility shift titrations in polyacrylamide gels by using ³²P-end-labelled T/CRE^{hs} showed that compound **2** was only weakly bound: even at a concentration of 200 nM it failed to saturate T/CRE^{hs} (Figure 2c, lanes 1–8), whereas near-saturation was achieved by just a 20 nM concentration of compound **1** (Figure 2b). Using as DNA probe ³²P-T/CRE^{hs}_m (Figure 2c, lanes 9–14) we observe the formation of a slightly slower migrating band which probably arises from a complex in which the peptide moiety is not folded inside the groove but electrostatically bound to the phosphate surface.

That **1**, in which the tether contains an NH group which is most probably protonated at the working pH, has a greater affinity for T/CRE^{hs} than **2**, in which the NH is replaced by an ether linkage, seems likely to be due to the NH group of **1** forming a salt bridge with phosphodiester oxygens in the section of oligonucleotide backbone straddled by the peptide–tether–tripyrrrole conjugate (see Figure 1). This conclusion is consistent with the notion that hydrogen bonds between DNA-binding proteins and DNA backbone phosphates, which account for about half of the direct hydrogen bonds observed in protein–DNA complexes,

contribute in a great measure to their association constants.^[12] In the case of our hybrid systems this type of interaction could even be more relevant for the stability of the complex owing to the special location of the amine group in the tether connecting the major and minor groove binding counterparts.

Design, synthesis and DNA-binding properties of a peptide–tripyrrrole derivative containing a propylamino chain in the middle pyrrole: The affinity of hybrid **1** for its specific target dsDNA decreases over 10-fold when the interaction

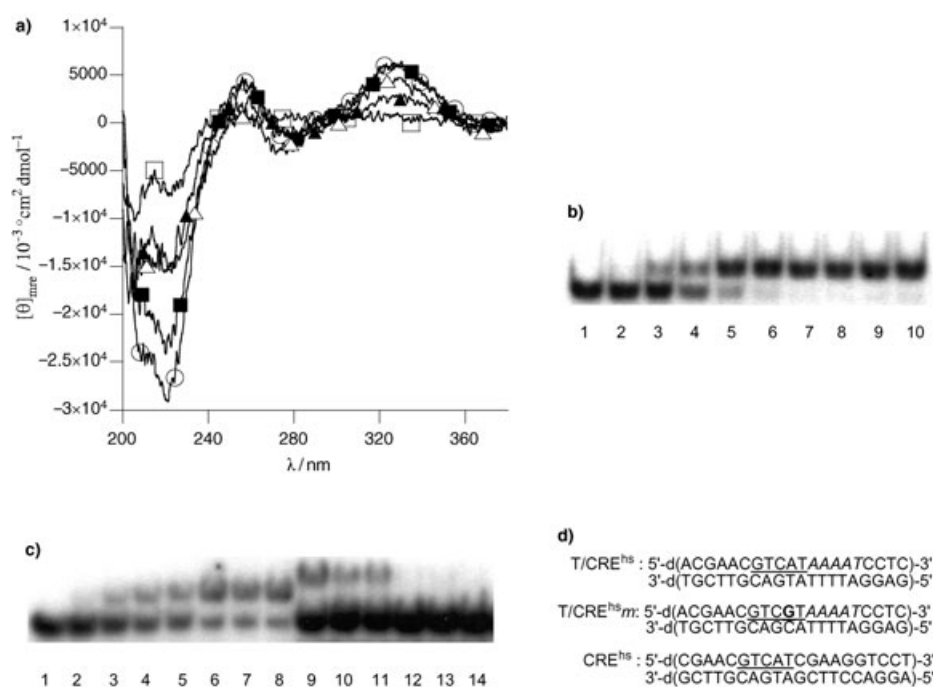


Figure 2. DNA-binding properties of **2**. a) CD difference spectra of hybrid **1** in the presence of T/CRE^{hs} (○), and of **2** in the presence or absence of ds-oligonucleotides: in the absence of DNA (□), in the presence T/CRE^{hs} (■), in the presence CRE^{hs} (▲), in the presence of T/CRE^{hs}m (△); CD spectra were obtained at 4°C as described in the Experimental Section, and were slightly smoothed to facilitate viewing. The difference spectra are the spectra of ligand + DNA mixture minus that of the DNA. b) Autoradiogram showing the binding of hybrid **1** to ³²P-labelled T/CRE^{hs}, lanes 1–10: 0, 1, 2, 5, 10, 20, 40, 60, 80, 100 nM. c) Autoradiograms showing the binding of hybrid **2** to ³²P-labelled DNAs, lanes 1–8, T/CRE^{hs}: 0, 20, 40, 60, 80, 100, 150, 200 nM; lanes 9–14: T/CRE^{hs}m: 200, 150, 100, 80, 40, 0 nM. d) Sequences of duplex oligonucleotides used. The BR subsite (CRE^{hs}) is underlined and the tripyrrole binding site (T) is in italics.

assay is carried out at room temperature instead of at 4°C. This is a major hurdle if one wants to develop this DNA recognition approach, based on simultaneous minor and major groove binding, into a viable strategy to compete with the DNA-binding ability of naturally occurring transcription factors. Therefore it would be very convenient and highly desirable to obtain derivatives that bind with low nanomolar dissociation constants at higher temperatures. The results described above, which support the hypothesis that the good affinity of **1** was in part due to its tether containing an amino group that interacted with the oligonucleotide backbone, suggested that affinity might be further increased by facilitating more ligand–backbone interactions of this kind. In view of the literature on related oligopyrroles,^[13] and after examining a simple qualitative working model of the conjugate–oligonucleotide complex (Figure 3), we decided to investigate the binding properties

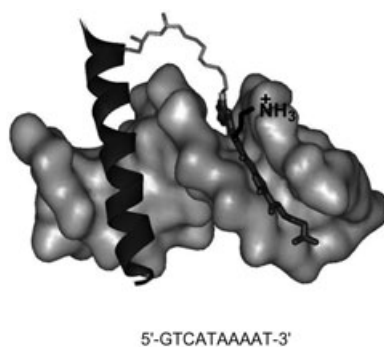
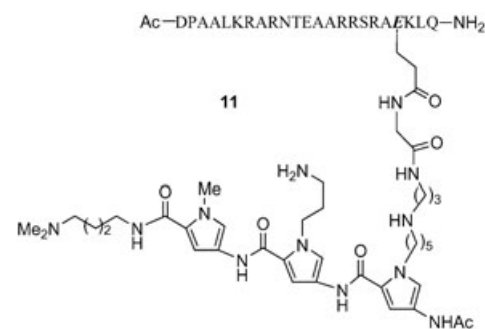


Figure 3.

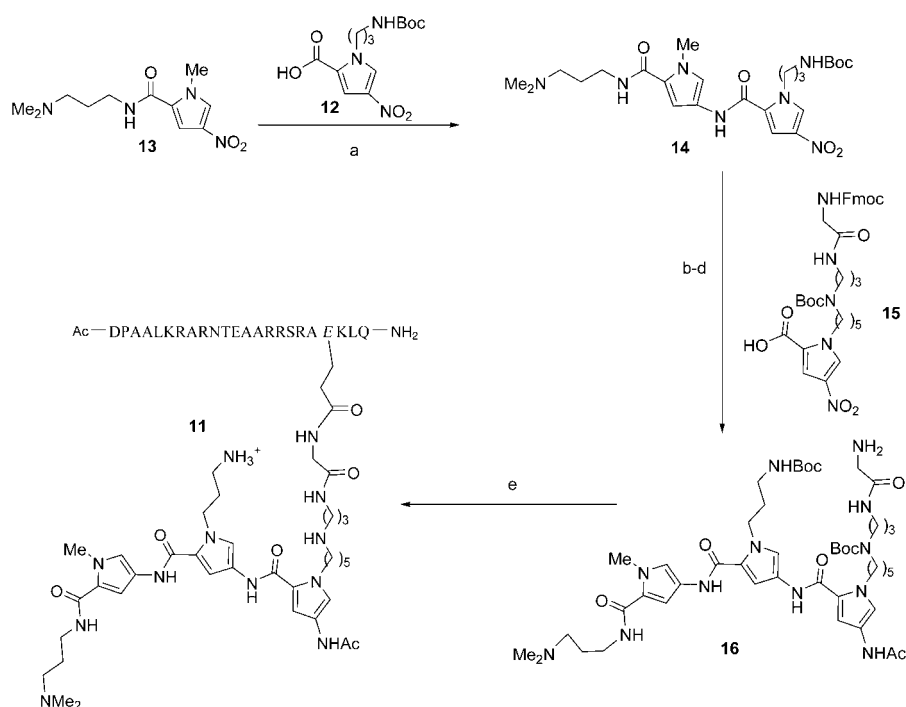


presence of the target DNA (T/CRE^{hs}) at 20°C revealed a slightly higher helicity than that observed for its relative **1** (Figure 4a).^[14] A similar pattern of relative intensities is exhibited by the band around 330 nm that reflects binding by the tripyrrole moiety. As could be expected, the CD spectra acquired in the presence of a duplex oligomer having a mutated peptide-binding half site (T/CRE^{hs}m) or lacking an AT rich sequence (CRE^{hs}) show a significantly weaker α-helical folding transition.

of compound **11**, in which an aminopropyl chain has been installed on the middle pyrrole of **1**.

Compound **11** was synthesized by linking the aminopropylated monopyrrole **12** to the terminal pyrrole **13** on one side and to the tether-bearing pyrrole **15** on the other, and then coupling the resulting tripyrrole peptide to the same GCN4 BR segment as in **2** (Scheme 3). An important aspect of the strategy was the use of “orthogonal” protecting groups for distinct terminal amines present in each of the N-pyrrole chains, so that it is possible to selectively unmask the amino group present in the tether of the N-terminal pyrrole. After TFA treatment to remove protecting groups and cleave the BR moiety from the resin, compound **11** was obtained in approximately 17% overall yield.

As shown in Figure 4a, the CD difference spectrum of the peptide conjugate **11** in the



Scheme 3. Synthesis of the peptide–tripyrrole hybrid **11**. a) 1) H_2 , Pd/C; 2) DECP, Et_3N , DMAP, THF, **12**; b) 1) H_2 , Pd/C; 2) DECP, Et_3N , DMAP, THF, **15**; c) 1) H_2 , Pd/C; 2) AcCl , DIEA; d) piperidine/DMF; e) 1) HATU, DIEA, DMF, **10**; 2) TFA, scavengers.

Gratifyingly, mobility shift titrations using ^{32}P -end-labelled T/CRE^{hs} showed that at 23 °C half-saturation with **11** occurred at a concentration of only about 5 nM, as against more than 50 nM for compound **1** at this temperature (Figure 4b and c).^[15] Like compound **2**, compound **11** also formed a less mobile complex with T/CRE^{hsm}, which we again attribute to non-specific binding without insertion of the BR moiety into the major groove; in this case half-saturation was achieved at a concentration of about 40 nM, near one order of magnitude greater than for the oligonucleotide with the full BR target sequence (Figure 4c, lanes 8–14). The affinity of **11** for CRE^{hs}, which lacks the tripyrrole-binding AAAAT motif, was negligible (Figure 4d). These electrophoresis results combined with the CD spectra confirm that the new hybrid peptide–tripyrrole derivative **11** is able to bind at room temperature (~23 °C) to designated dsDNA sequences with low nanomolar dissociation constants, being enormously selective with respect to sequences that lack an AT rich region and almost 10-fold more selective with regard to mismatch DNA sites containing this type of regions.

Conclusion

There is a great deal of interest in obtaining artificial mimics of transcription factors that being smaller in size are yet capable of reproducing their DNA-binding specificity and affinity. It is desirable that these artificial DNA-binding

peptides are able to address sequences containing relatively high number of bases, so they could target particular sites in the genome with higher specificity. These molecules, in addition to providing basic information about the factors that govern sequence-specific protein–DNA interactions, might find important biomedical applications as designed genome interference agents.

Building on previous work in which monomeric bZIP BRs had been found capable of low-temperature specific DNA recognition when linked by a tether to tripyrroles capable of minor-groove binding to flanking sequences, in this work we investigated the contributions to binding affinity made by the tether and by an appropriate tripyrrole side chain. High-affinity binding was found to require the presence in the tether of a suitably placed secondary

amino group, probably because it forms a salt bridge with phosphates in the segment of DNA backbone straddled by the tether. This conclusion led us to design a new peptide–tripyrrole hybrid deliberately equipped with an aminopropyl residue in one of the pyrroles, anticipating that it might exhibit higher DNA affinities owing to the generation of similar kind of electrostatic contacts. Addition of the tripyrrole moiety to an aminopropyl side chain capable of similar interactions resulted in compound **11** which achieves low nanomolar affinity for the composite consensus half site T/CRE^{hs} even at room temperature (approximately 10-fold improvement with respect to the analogue lacking this side chain). As far as we know, compound **11** is the smallest artificially designed transcription-factor-based peptide derivative to have proved capable of recognizing such long DNA sequences (9 or 10 bp) with such high affinity. Work to further improve the design process so that in addition to high affinity we can also attain even better specificities, as well as to obtain more structural and thermodynamic data to further characterize these DNA–peptide complexes, is under way.

Experimental Section

General procedures and protocols: All dry solvents were freshly distilled under argon from the appropriate drying agent before use. THF and Et_2O were distilled from sodium/benzophenone. CH_2Cl_2 was distilled from CaH_2 . All reactions were conducted in dry solvents under argon at-

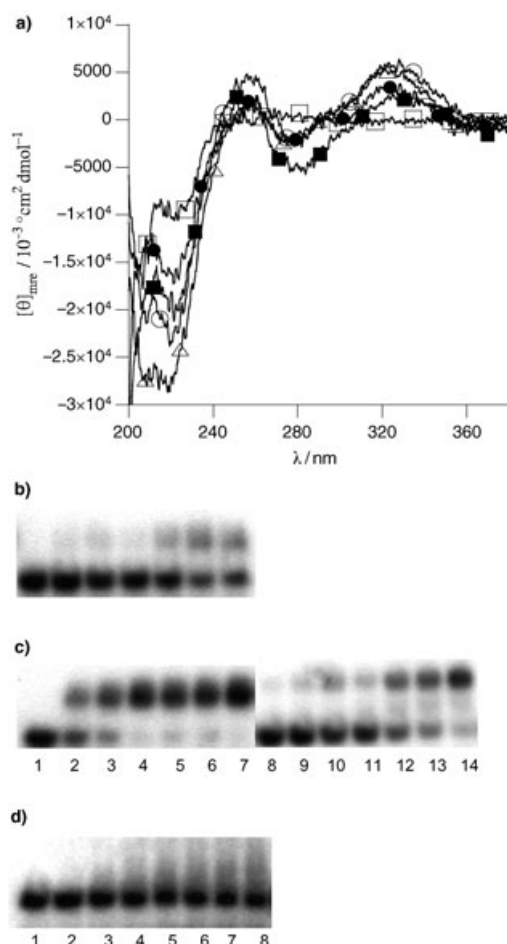


Figure 4. DNA-binding properties of **11**. a) CD difference spectra of hybrid **1** in the presence of T/CRE^{hs} (○) and of peptide **11** in the presence or absence of ds-oligonucleotides: in the absence of DNA (□), in the presence T/CRE^{hs} (△), in the presence CRE^{hs} (●), in the presence of T/CRE^{hs}m (■); CD spectra were obtained at 20°C as described in the Experimental Section. The contribution of the DNA has been subtracted. b) Autoradiogram showing the binding of hybrid **1** to ³²P-labelled T/CRE^{hs} at 23°C: lanes: 5, 10, 15, 20, 30, 40, 50 nm. c) Autoradiograms showing the binding of hybrid **11** to ³²P-labelled DNAs at 23°C: lanes 1–7: T/CRE^{hs}: 0, 5, 10, 15, 20, 30, 40 nm; lanes 8–14: T/CRE^{hs}m: 5, 10, 15, 20, 30, 40, 50 nm. d) Autoradiogram showing the binding of hybrid **11** to ³²P-labelled CRE^{hs} at 23°C: 0, 100, 200, 300, 400, 500, 600, 700 nm.

mosphere unless otherwise stated. Thin-layer chromatography (TLC) was performed on silica gel plates and components were visualized by observation under UV light, or by treating the plates with a *p*-anisaldehyde, phosphomolybdic or ninhydrin reagent followed by heating. Drying was performed with anhydrous Na₂SO₄ and concentrations were carried out in a rotary evaporator. ¹H and ¹³C NMR spectra were recorded in CDCl₃, at 250 MHz and 62.9 MHz, respectively, and in some cases at 300 or 500 MHz (75.4 or 125.7 for ¹³C NMR). Carbon types were determined from DEPT and ¹³C NMR experiments.

Peptide synthesis was performed using standard Fmoc solid phase synthesis method on a Rink-MBHA amide resin (~0.46 mmol g⁻¹), using mixtures of HBTU/HOBt as coupling agents, DIEA as base and DMF as solvent. The cleavage/deprotection step was performed by treatment of the resin-bound peptide with the following mixture: 830 μL TFA, 25 μL EDT, 50 mg PhOH, 50 μL tioanisole and 50 μL H₂O (300 μL of this mixture for each 10 mg of resin). The amino acids used in the synthesis were

standard protected, except the Fmoc-Glu(OAll)-OH introduced at position 245.

CD measurements were made in a 2 mm cell. Samples contained 10 mM phosphate buffer (pH 7.5), 100 mM NaCl, 5 μM peptide and 5 μM ds-oligo when present. The peptide–DNA mixtures were incubated for 15 min before registering. The spectra are the average of 5 scans and were slightly smoothed using the “smooth” macro implemented in the program Kaleidagraph (v 3.5 by Synergy Software). Spectra of the peptides in the presence of DNA were calculated as the difference between the spectra of the peptide–DNA mixture and the spectrum of free DNA.

For gel mobility shift assays, binding reactions were performed over 30 min by using ~45 pM labeled dsDNAs (unless otherwise stated) in a binding mixture (20 μL) containing 18 mM Tris (pH 7.5), 90 mM KCl, 1.8 mM MgCl₂, 1.8 mM EDTA, 9% glycerol, 0.3 mg mL⁻¹ BSA and 2.2% NP-40. Products were resolved by PAGE by using a 10% nondenaturing acrylamide gel and 0.5X TBE buffer, and analyzed by autoradiography (TBE 0.5X buffer is 44 mM boric acid, 44 mM Tris base and 0.1 mM EDTA pH 8).

Synthesis of the tripyrrole derivative **8**

{[3-[5-(4-Acetylaminopropyl)-5-[5-[3-(dimethylaminopropyl)carbamoyl]-1-methyl-1H-pyrrol-3-yl-carbamoyl]-1-methyl-1H-pyrrol-3-yl-carbamoyl]-pyrrol-1-yl]-pentoxyl-propyl-carbamic acid *tert*-butyl ester: K₂CO₃ (330 mg) and iodide **3** (299 mg, 0.9 mmol) were added to a solution of tripyrrole **9** (100 mg, 0.2 mmol) in dry acetone (6 mL). The reaction mixture was refluxed for 8 h and the resulting suspension was filtered through Celite. The filtrate was concentrated and the residue purified by flash chromatography (neutral aluminium oxide, 5% MeOH/CH₂Cl₂) to afford the expected product as a pale-yellow solid. ¹H NMR (CD₃OD): δ = 1.25–1.45 (brs, 13H), 1.52–1.58 (m, 2H), 1.62–1.70 (m, 4H), 2.00–2.05 (m, 5H), 3.04 (brs, 10H), 3.22–3.38 (m, 6H), 3.83–3.86 (2 s, 6H), 6.92–6.98 (m, 3H), 7.19–7.22 (m, 3H); ¹³C NMR (CD₃OD): δ = 23.1 (CH₃), 23.4 (CH₂), 24.2 (CH₂), 24.4 (CH₂), 28.8 (CH₃), 30.1 (CH₂), 31.1 (CH₂), 36.9 (CH₃), 38.9 (CH₂), 49.9 (CH₃), 51.5 (CH₃), 63.1 (CH₂), 65.3 (CH₂), 69.4 (CH₂), 71.4 (CH₂), 79.8 (C), 104.0 (CH), 106.6 (CH), 114.4 (CH), 120.8 (CH), 123.2 (C), 123.4 (C), 124.0 (C), 124.5 (C), 124.6 (C), 125.3 (C), 158.4 (C), 160.5 (C), 161.2 (C), 164.3 (C), 170.3 (C); MS (FAB+): *m/z*: 740 (36) [M+H]⁺, 618 (14); HRMS: *m/z*: calcd for C₃₇H₅₈N₉O₇: 740.4459, found 740.4459.

{[3-[5-(4-Acetylaminopropyl)-5-[5-[3-(dimethylaminopropyl)carbamoyl]-1-methyl-1H-pyrrol-3-yl-carbamoyl]-1-methyl-1H-pyrrol-3-yl-carbamoyl]-pyrrol-1-yl]-pentoxyl-propyl-carbamic acid (8**)**: The above-mentioned tripyrrole derivative (150 mg, 0.2 mmol) was dissolved in CH₂Cl₂ (3 mL) and cooled to 0°C. TFA (3 mL) was added dropwise and the resulting orange solution was stirred at 0°C for 1 h and at room temperature for another 2 h. The solvents were removed under reduced pressure and the residual TFA was removed by codistillation with CH₂Cl₂. The resulting residue was dissolved in DMF (5 mL) and to this solution was added DIEA (0.2 mL, 0.98 mmol) and Boc-Gly-OSu (54 mg, 0.20 mmol). The resulting solution was stirred for 1 h at room temperature, the solvent evaporated and the residue purified by flash chromatography (neutral aluminium oxide, 5% MeOH/CH₂Cl₂) and submitted to the standard protocol for removal of the Boc protecting group (TFA/CH₂Cl₂). The residue resulting from removal of the solvents consisted of the desired tripyrrole **8** (43 mg, 31%). ¹H NMR (CD₃OD): δ = 1.15 (m, 4H), 1.39 (m, 2H), 1.57 (m, 4H), 1.83 (m, 2H), 1.93 (s, 3H), 2.74 (s, 6H), 3.01 (t, *J* = 7.3 Hz, 2H), 3.12 (t, *J* = 6.9 Hz, 2H), 3.20–3.30 (m, 4H), 3.50 (s, 2H), 3.72–3.74 (2 s, 6H), 4.16 (t, *J* = 6.3 Hz, 2H), 6.67 (s, 1H), 6.72 (s, 2H), 6.79 (s, 2H), 7.01 (s, 1H); ¹³C NMR (CD₃OD): δ = 22.9 (CH₃), 24.3 (CH₂), 26.4 (CH₂), 30.3 (CH₂), 30.4 (CH₂), 32.7 (CH₂), 36.7 (CH₂), 36.8 (CH₃), 36.9 (CH₃), 38.0 (CH₂), 41.5 (CH₂), 43.4 (CH₃), 49.6 (CH₂), 56.6 (CH₂), 69.1 (CH₂), 71.7 (CH₂), 106.2 (CH), 106.6 (CH), 119.6 (CH), 120.9 (CH), 123.2 (C), 123.9 (C), 124.0 (C), 124.5 (C), 161.4 (C), 161.5 (C), 164.9 (C), 167.2 (C), 170.4 (C); MS (FAB+): *m/z*: 697 (100) [M+H]⁺; HRMS: *m/z*: calcd for C₃₄H₅₃N₁₀O₆: 697.4150, found 697.4151.

Synthesis of the tripyrrole derivative **16**

{[2-[5-(3-Aminopropylcarbamoyl)-1-methyl-1H-pyrrol-3-yl-carbamoyl]-4-nitropyrrol-1-yl]-propyl-carbamic acid *tert*-butyl ester (14**)**: A solution of nitropyrrole **13** (615 mg, 2.42 mmol) in MeOH (30 mL) was hydrogen-

ated over 10% palladium on charcoal (400 mg) at room temperature for 1.5 h (balloon pressure). The catalyst was removed by filtration through Celite, and the filtrate concentrated under reduced pressure. The residue was dissolved in DMF (8 mL) and added over another solution of nitroacid **12** (584 mg, 1.86 mmol), DECP (0.366 mL, 2.42 mmol), Et₃N (1.23 mL, 9.3 mmol) and DMAP (10 mg) in THF (25 mL) cooled at 0°C. The resulting mixture was allowed to stir at room temperature for 8 h and concentrated under reduced pressure. The product was purified by flash column chromatography in neutral aluminium oxide (5% MeOH/CH₂Cl₂) to afford **14** (870 mg, 90%). ¹H NMR (CDCl₃): δ = 1.41 (s, 9H), 1.75 (q, *J* = 7.2 Hz, 2H), 1.96 (q, *J* = 6.7 Hz, 2H), 2.25 (s, 6H), 2.39 (m, 2H), 3.04 (t, *J* = 6.6 Hz, 2H), 3.30 (m, 2H), 3.84 (s, 3H), 4.44 (t, *J* = 6.8 Hz, 2H), 6.77 (s, 1H), 7.19 (s, 1H), 7.38 (s, 1H), 7.90 (s, 1H); ¹³C NMR (CDCl₃): δ = 28.2 (CH₂), 28.8 (CH₃), 32.6 (CH₂), 36.8 (CH₃), 38.4 (CH₂), 38.7 (CH₂), 45.6 (CH₃), 48.6 (CH₂), 58.4 (CH₂), 80.1 (C), 105.8 (CH), 109.1 (CH), 120.4 (CH), 122.9 (C), 124.7 (C), 127.2 (CH), 128.0 (C), 136.4 (C), 158.4 (C), 159.4 (C), 164.0 (C); MS (FAB+): *m/z*: 520 (100) [*M*+H]⁺, 420 (5); HRMS: *m/z*: calcd for C₂₄H₃₈N₇O₆ 520.2884, found 520.2904.

[5-(4-Acetylamino-2-[1-(3-*tert*-butoxycarbonylamino)propyl]-5-[5-(3-dimethylamino-propylcarbamoyl)-1-methyl-1H-pyrrol-3-yl-carbamoyl]-1H-pyrrol-3-yl-carbamoyl]-pyrrol-1-yl)-pentyl]-[3-(2-aminoacetylaminopropyl)-carbamoyl]-carbamoyl acid *tert*-butyl ester (16**):** A solution of the dipyrrole **14** (300 mg, 0.58 mmol) in MeOH (30 mL) was hydrogenated over 10% palladium on charcoal (200 mg) at room temperature for 1.5 h (balloon pressure). The catalyst was removed by filtration through Celite, and the filtrate concentrated under reduced pressure. The residue was dissolved in DMF (8 mL) and added over another solution previously prepared of **15** (510 mg, 0.75 mmol), DECP (0.123 mL, 0.81 mmol), Et₃N (0.402 mL, 2.9 mmol) and DMAP (10 mg) in THF (20 mL) cooled at 0°C. The resulting mixture was allowed to stir at room temperature for 8 h and concentrated under reduced pressure. The product was purified by flash column chromatography in neutral aluminium oxide (5% MeOH/CH₂Cl₂) to afford the expected nitrotripyrrole (439 mg, 66%). ¹H NMR (CD₃OD): δ = 1.17–1.22 (m, 2H), 1.33–1.36 (2 s, 18H), 1.36–1.40 (m, 2H), 1.48–1.63 (m, 2H), 1.70–1.75 (m, 2H), 1.83–1.94 (m, 4H), 2.84 (s, 6H), 2.96 (t, *J* = 6.6 Hz, 2H), 3.06–3.12 (m, 8H), 3.33 (t, *J* = 6.3 Hz, 2H), 3.68 (s, 2H), 3.81 (s, 3H), 4.12 (t, *J* = 6.7 Hz, 1H), 4.26–4.38 (m, 6H), 6.83 (s, 1H), 6.92 (s, 1H), 7.13 (s, 1H), 7.21–7.34 (m, 6H), 7.54 (s, 1H), 7.57 (s, 1H), 7.69 (s, 1H), 7.72 (s, 1H), 7.84 (s, 1H); ¹³C NMR (CD₃OD): 24.6 (CH₂), 26.6 (CH₂), 28.8 (CH₃), 32.2 (CH₂), 33.0 (CH₂), 36.5 (CH₂), 37.0 (CH₃), 38.7 (CH₂), 43.5 (CH₃), 45.1 (CH₂), 47.2 (CH₂), 48.2 (CH₂), 50.8 (CH₂), 56.6 (CH₂), 68.2 (CH₂), 80.0 (C), 81.0 (C), 106.7 (CH), 109.2 (CH), 119.8 (CH), 121.0 (CH), 123.3 (C), 123.8 (C), 124.3 (C), 126.2 (CH), 127.3 (C), 128.0 (CH), 128.2 (CH), 128.9 (CH), 136.4 (C), 142.6 (C), 145.3 (C), 158.5 (C), 159.1 (C), 159.6 (C), 161.2 (C), 165.0 (C), 172.3 (C); HRMS (ESI-TOF): *m/z*: calcd for C₅₉H₈₁N₁₂O₁₂ 1149.6097, found 1149.609.

A solution of this nitrotripyrrole (490 mg, 0.427 mmol) in MeOH (40 mL) was hydrogenated for 1 h over 10% palladium on charcoal (250 mg) at room temperature (balloon pressure). The catalyst was removed by filtration through celite and the filtrate was concentrated. The residue was immediately dissolved in THF (10 mL) containing DIEA (0.367 mL, 2.14 mmol) and cooled to 0°C. AcCl (0.152 mL, 2.14 mmol) was added and the solution was stirred at 0°C for 30 min and at room temperature for 3 h. The solvents were evaporated under vacuum and the resulting residue was purified by flash chromatography (aluminium oxide, 5% MeOH/CH₂Cl₂) to give the desired acetylated derivative as a brown solid (317 mg, 64%). ¹H NMR (CD₃OD): δ = 1.06–1.25 (m, 4H), 1.35–1.37 (2 s, 18H), 1.52–1.74 (m, 4H), 1.82–1.95 (m, 4H), 2.02 (s, 3H), 2.85 (s, 6H), 2.97 (t, *J* = 6.3 Hz, 2H), 3.01–3.17 (m, 8H), 3.25–3.28 (m, 2H), 3.35 (t, *J* = 5.7 Hz, 2H), 3.70 (s, 2H), 3.82 (s, 3H), 4.13 (t, *J* = 6.9 Hz, 1H), 4.28–4.30 (m, 4H), 6.78–6.92 (m, 3H), 7.14–7.25 (m, 5H), 7.32 (t, *J* = 7.3 Hz, 2H), 7.55 (s, 1H), 7.58 (s, 1H), 7.71 (s, 1H), 7.74 (s, 1H); HRMS (ESI-TOF): *m/z*: calcd for C₆₁H₈₅N₁₂O₁₁ 1161.6461, found 1161.642.

Piperidine (2 mL) was added to a solution of the above Fmoc-containing tripyrrole (295 mg, 0.254 mmol) in CH₂Cl₂ (8 mL) and the resulting solu-

tion was stirred at room temperature for 30 min. Solvents were removed and the residue was purified by HPLC (gradient 5 → 95% B, *t*_R = 30 min; A: TFA/H₂O 0.1%, B: TFA/CH₃CN 0.1%) to afford the desired product **16** (165 mg, 69%). ¹H NMR (CD₃OD): δ = 1.22–1.35 (m, 4H), 1.43–1.44 (2 s, 18H), 1.55–1.60 (m, 2H), 1.70–1.89 (m, 4H), 1.94 (t, *J* = 6.6 Hz, 2H), 2.08 (s, 3H), 3.09 (s, 6H), 3.15–3.41 (m, 14H), 3.66 (s, 2H), 3.89 (s, 3H), 4.38 (t, *J* = 6.41 Hz, 2H), 6.90 (s, 1H), 6.93 (s, 1H), 6.98 (s, 1H), 7.19 (s, 1H), 7.20 (s, 1H), 7.26 (s, 1H); MS (FAB+): *m/z*: 939 (9) [*M*+H]⁺, 839 (3), 739 (10); HRMS: *m/z*: calcd for C₄₆H₇₅N₁₂O₉: 939.5780, found 939.5771.

Synthesis of the peptide–tripyrrole hybrids: The following is the general procedure for the coupling of the amino tripyrrole derivatives with the solid-phase bound peptide, exemplified for the coupling of tripyrrole **8** to the resin-linked peptide **10** to give hybrid **2**. Resin-bound peptide **10** (25 mg, Eppendorf tube) was suspended in DMF (1 mL) and mixture was shaken for 1 h to ensure a good resin swelling. The DMF was removed a solution of HATU in DMF (2.6 mg in 170 μL) and DIEA (28 μL, 0.5 M in DMF) was added. The resulting mixture was shaken for 5 min, and a solution of the tripyrrole **8** (10 mg in 70 μL of DMF) and 28 μL of DIEA (0.5 M in DMF) was added. The reaction mixture was shaken for 2 h, and the resin washed with DMF (3 × 0.6 mL, for 5 min), and Et₂O (2 × 0.5 mL). Cleavage/deprotection of the bound peptide under standard conditions afforded a major product that was purified by RP-HPLC (gradient 10 → 35%, *t*_R = 24.32 min). MALDI-MS analysis confirmed the formation of the desired hybrid **2** (~36% yield, considering also the peptide synthesis): *m/z*: calcd for C₁₄₃H₂₄₄N₅₃O₃₉: 3327.9, found 3327.4 [*M*+H]⁺; **1**: [~26% yield]; MALDI-TOF: calcd for C₁₄₃H₂₄₅N₅₄O₃₈: 3326.9, found 3326.6 [*M*+H]⁺; RP-HPLC: *t*_R = 23.38 min; **11**: [~17% yield]; MALDI-TOF: calcd for C₁₄₅H₂₅₀N₅₅O₃₈: 3369.9, found 3370.0 [*M*+H]⁺; RP-HPLC: *t*_R = 21.0 min.

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- [14] At 4 °C the $[\theta]_{\text{mre}}$ at 222 nm has an approximate value of $-30000^\circ\text{cm}^2\text{dmol}^{-1}$, slightly higher than that observed for **1** complexed to the same dsDNA.
- [15] At 4 °C **11** binds the target dsDNA with subnanomolar dissociation constants. See gel shift titration in the Supporting Information.

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