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Guanine-Containing DNA Minor-Groove Binders

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Solid-phase procedures have been used to prepare six dipyrrole-containing DNA ligands that combine (guanin-9-yl)-acetyl, (guanin-7-yl)acetyl or acetyl moieties at the N-terminal end and two lysines or a (dimethylamino)propyl group at the C terminus. Inspection of their DNA-stabilizing properties by UV-monitored thermal denaturation experiments showed that the ligand incorporating the (guanin-9-yl)acetyl group and the (dimethylamino)propyl tail had the highest duplex-stabilizing effects.

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Introduction

The past 30 years have witnessed outstanding progress in the design and preparation of minor-groove binders with improved properties for the control of gene expression. The first studies that suggested an interaction between netropsin and the minor groove of DNA[1] were followed by NMR[2] and X-ray[3] structure analyses of netropsin–DNA complexes, and these by many other studies addressing chemical, structural and biological issues. To mention just a few, the replacement of pyrrole by imidazole rings (lexitropsins) extended the interaction of minor-groove binders from A·T to G·C pairs.[4] Lexitropsins were combined with either alkylating or intercalating agents[5] to afford compounds with antitumour properties. In microgonotropens, the substitution of polyamines for methyl groups on pyrrole nitrogen atoms allowed additional electrostatic interactions with the phosphates to be established, thus increasing the affinity for the target.[6] Minor-groove binders have also been combined between themselves,[7] as well as with peptides[8] and oligonucleotides.[9] Finally, with the polyamides developed by Dervan and co-workers, the specific recognition of DNA sequences has become a reality, because the four natural base pairs can be distinguished by using the right combination of different heterocyclic rings.[10]

Over the years, the number of solved structures of oligonucleotides, either in the presence or in the absence of drugs, has continued to increase. One interesting feature that has become very evident from all this research is the ubiquity of guanine,[11] which can interact with other guanines and with different nucleobases in many ways. It is well known that guanines can form G-tetrads and recognize G·C pairs in antiparallel triplex structures, in addition to base-pairing with cytosines.

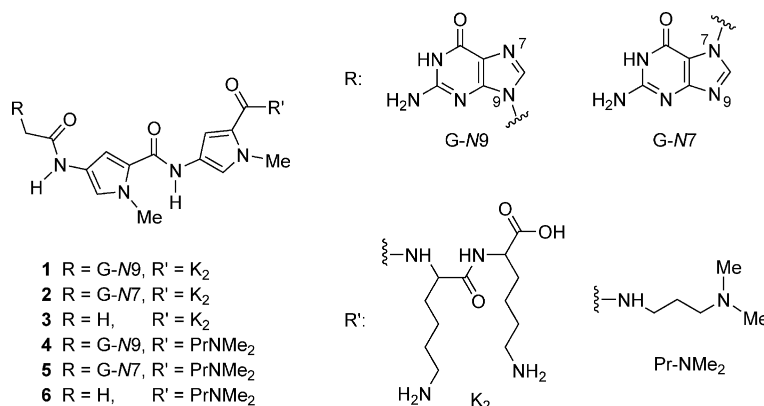

Figure 1. Structures of the pyrrole-containing DNA ligands.

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The potential of guanines to interact with other guanines within the minor groove through hydrogen bonds involving the N-3 atom and the exocyclic amine is poorly recognized,[11] and guanines have not been linked to minor-groove binders. To the best of our knowledge, only minor-groove binders incorporating adenine, thymine and uracil have been synthesized and their properties evaluated.[12] In this manuscript we wish to describe the synthesis of four guanine–pyrrole conjugates and an assessment of their DNA binding affinities.

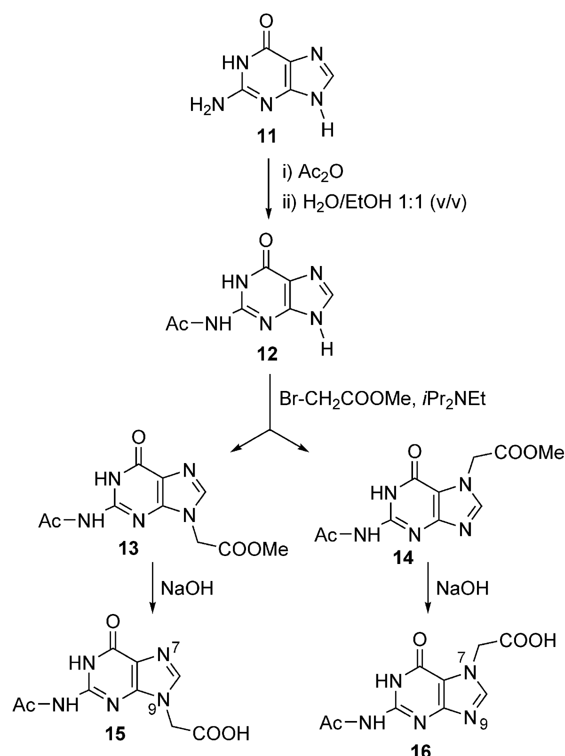
The DNA ligands **1**, **2**, **4** and **5** shown in Figure 1 were designed to incorporate two *N*-methylpyrrole units, as in netropsin, the guanine moiety linked through either the N-7 or N-9 atom, and a positive charge at the C-terminal end, which was provided by the presence of either two lysines or a (dimethylamino)propyl group. As a stronger interaction with DNA results in increased duplex stability,[13] their potential as minor-groove binders was assessed from their duplex-stabilizing properties, which was evaluated through UV-monitored thermal denaturation experiments. For comparison purposes, ligands with an acetyl group replacing the guanine building block (**3** and **6**) have also been prepared and studied.

Results and Discussion

All the molecules were assembled by using solid-phase technologies, which seemed the most convenient way to achieve a quick access to the different conjugates and thus allow their properties to be evaluated. Both the pyrrole and guaninylacetyl building blocks have been synthesized previously.

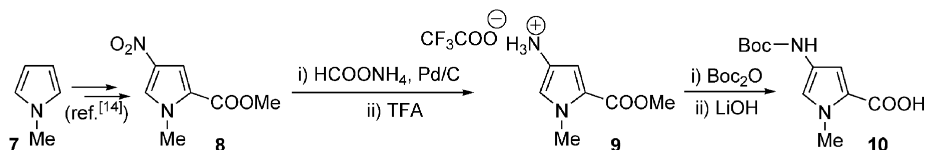
The *N*-Boc-protected (Boc = *tert*-butoxycarbonyl) *N*-methylpyrrole derivative **10** was prepared according to literature procedures[14] with minor modifications (Scheme 1). In our hands, treatment of methyl *N*-methyl-4-nitropyrrole-2-carboxylate (**8**) with H_2 in the presence of Pd/C followed by bubbling with hydrogen chloride did not afford the desired amine derivative. Instead, reduction of **8** with ammonium formate in the presence of Pd/C followed by the addition of trifluoroacetic acid gave the trifluoroacetate salt of methyl *N*-methyl-4-aminopyrrole-2-carboxylate (**9**). Other authors have experienced problems in this synthesis step,[15] and an alternative strategy for the preparation of the pyrrole building block that did not include the nitro-to-amine reduction has recently been described.[16] Protection of the amine with the Boc group and ester hydrolysis proceeded smoothly. Note that these minor changes allowed **10** to be obtained from **8** in a yield of 83%.

To prepare the guaninylacetic acids **15** and **16** (Scheme 2), the exocyclic amine of guanine was first protected by acetylation. Then reaction of N^2-acetylguanine (**12**) with methyl bromoacetate and ethyldiisopropylamine afforded, as expected, a mixture of two isomeric methyl acetates resulting from guanine alkylation at either the N-9 (**13**) or N-7 (**14**) atom. These compounds were separated by fractional crystallization and, after their structural assignment (see below), the ester was hydrolysed by reaction with sodium hydroxide.



Scheme 2. Preparation of guaninylacetic acids **15** and **16**.

Identification of the N^7 and N^9 guanine regioisomers was first based on differences in their UV spectra and, later, differences in chemical shifts between the two isomers.[17] However, unequivocal assignment on this basis is reliable only if the two isomers are available and the corresponding spectroscopic data can be compared. We have made use of two-dimensional heteronuclear correlation HMBC experiments,[18] which allow the two isomers to be unequivocally identified. A signal correlating the CH_2 group (^1H chemical shift) and the C-5 atom of guanine (^{13}C chemical shift) appears in the spectrum of the N^7 isomer. Conversely, the CH_2



Scheme 1. Synthesis of (4-Boc-amino)-*N*-methylpyrrole-2-carboxylic acid (**10**).

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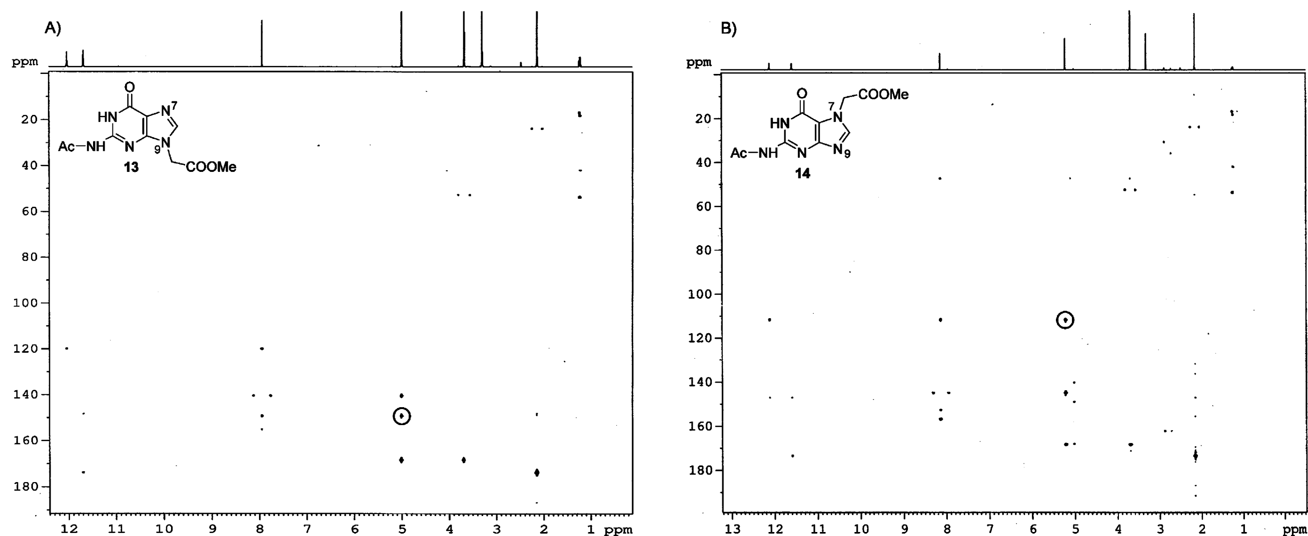
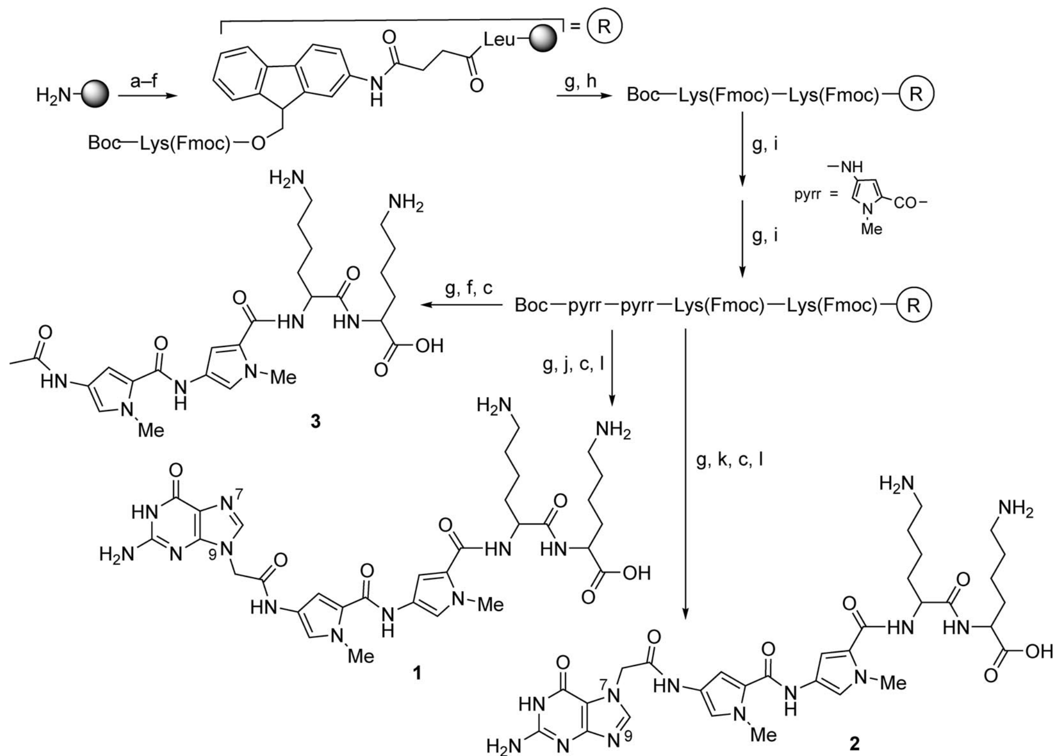


Figure 2. [^{1}H-^{13}C] HMBC NMR spectral characterization of (A) the N^9- (**13**) and (B) the N^7-alkylated (**14**) guanines. Circles correspond to the diagnostic CH_2–C4 and CH_2–C5 correlation signals, respectively.

group of the N^9 isomer is closer to C-4, and this is the correlation signal observed. The two spectra are shown in Figure 2 (see also the Exp. Sect.). This technique has been used to identify histidine tautomers,[19] but we are aware of only one case, in which it was used to differentiate guanine isomers.[20]

Compounds **1**–**3** were assembled as in standard solid-phase peptide synthesis by using a set of base-labile permanent protecting groups and acid-labile Boc temporary protecting groups (Scheme 3). Subsequent DCC-mediated (DCC = *N*,*N′*-dicyclohexylcarbodiimide) incorporation onto the *p*-methylbenzhydrylamine-polystyrene resin of an



Scheme 3. Solid-phase assembly and deprotection of pyrr$_2$-Lys$_2$ ligands **1**, **2** and **3**. (a) Fmoc-L-Leu-OH, DCC; (b) Ac_2O/DIEA; (c) 20% piperidine/DMF; (d) *N*-[9-(hydroxymethyl)-2-fluorenyl]succinamic acid (ref.[21]), DCC; (e) Boc-L-Lys(Fmoc)-OH, DCC, DMAP; (f) Ac_2O, pyr; (g) 40% TFA/DCM, followed by 5% DIEA/DCM; (h) Boc-L-Lys(Fmoc)-OH, DCC; (i) **10**, DIPC, DMAP; (j) **15**, DIPC, HOAt; (k) **16**, DIPC, HOAt; (l) concd. aq. NH_3.

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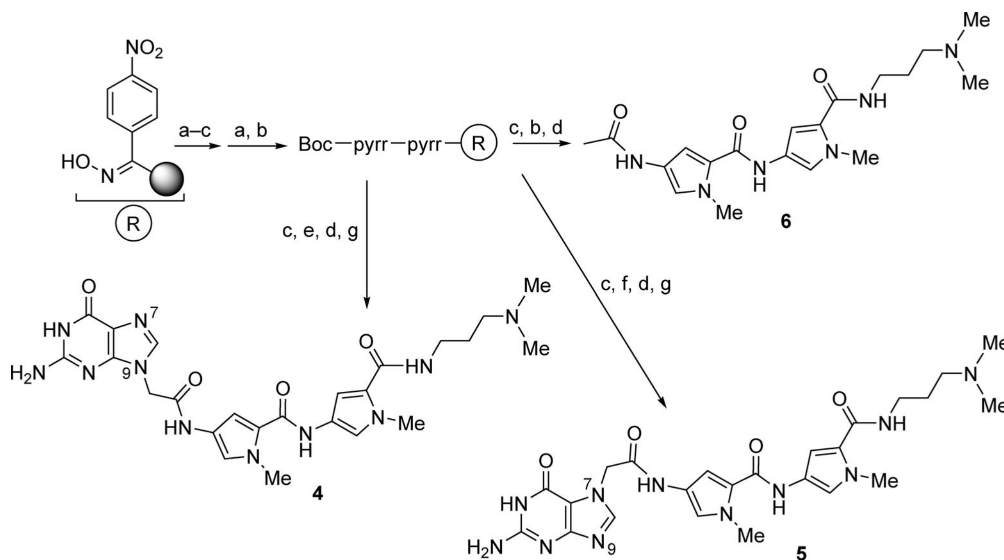
internal reference amino acid (leucine) and the *N*-[9-(hydroxymethyl)-2-fluorenyl]succinamic acid bifunctional linker[21] was followed by coupling of the two lysines [4-(dimethylamino)pyridine (DMAP) was added to form the lysine-bifunctional linker ester bond]. Different alternatives were examined for the incorporation of the pyrrole derivatives. The carboxy group of **10** was activated with carbodiimides [DCC or *N*,*N*′-diisopropylcarbodiimide (DIPC)] in the presence of different catalysts [DMAP, 1-hydroxybenzotriazole (HOBt) or 1-hydroxy-7-azabenzotriazole (HOAt)], and the use of activating reagents such as PyBOP [benzotriazol-1-yl-*N*-oxytris(pyrrolidino)phosphonium hexafluorophosphate] or HATU [2-(7-aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] was also tested. The best results were obtained by using 2:2:1 mixtures of **10**/DIPC/DMAP and a five-fold excess of **10**. After dividing the resin into three different batches, the Boc group was removed, and the amine was either acetylated or treated with the corresponding guaninylacetic acid derivative. Activation of the carboxy group of **15** (or **16**) with DIPC and HOAt was more effective than use of DIPC/DMAP or DIPC/HOBt mixtures. Finally, treatment with 20% piperidine in DMF (*N*,*N*-dimethylformamide) caused the conjugates to detach from the resin and removed the Fmoc groups protecting the lysine side-chains to yield **3** and partially protected **1** and **2**. Reaction with concd. aq. ammonia removed the acetyl group from the exocyclic amino group of the guanine and afforded crude **1** and **2**. All products were purified by reversed-phase HPLC and characterized by MALDI-TOF MS.

Compounds **4**–**6** were assembled on a Kaiser oxime resin,[22] as shown in Scheme 4. For incorporation onto the solid matrix, activation of the carboxy groups of the two pyrroles and the guanine building blocks was carried out as described above. The main differences with respect to the previous syntheses are that the coupling time of the first pyrrole unit had to be extended to 15 h and that Boc groups were removed by using a 20% TFA/DCM (TFA = trifluoroacetic acid, DCM = dichloromethane) solution rather than the standard 40% TFA-containing one that might cleave the polyamide-resin oxime ester bond. In addition, after all the coupling steps, the resin was washed with NMP, DCM and 2-propanol instead of simply with DCM (or DCM and DMF). Treatment with *N*,*N*-dimethylpropane-1,3-diamine led to the detachment of the conjugates from the solid support and introduced the cationic unit at the C terminus. Fully deprotected **4** and **5** were obtained after treatment with concd. aq. ammonia, which removed the acetyl group protecting the exocyclic amino group of guanine. Compounds **4**–**6** were purified and characterized as indicated above.

The two oligonucleotide chains $^{5'}$GTGAATTCTG$^{3'}$ and $^{5'}$CAGAATTCAC$^{3'}$ were chosen to yield a duplex with a central A/T-rich region flanked by G·C base pairs. Oligonucleotides were prepared by using standard procedures, namely solid-phase synthesis and phosphite triester chemistry, followed by ammonia treatment, medium-pressure reversed-phase purification and characterization by MALDI-TOF MS.

Then, to obtain information on the effect of combining guanine and pyrroles on the interaction with DNA, as well as the effect of using either of the guanine regioisomers, UV-monitored thermal denaturation experiments were carried out at two different ligand/DNA ratios. The results of these experiments are summarized in Table 1. Both the T_m and ΔT_m (difference between the T_m of the ligand–DNA complex and the T_m of the duplex) values are shown for comparison purposes.



Scheme 4. Solid-phase assembly and deprotection of pyrr$_2$-NMe$_2$ ligands **4**, **5** and **6**. (a) **10**, DCC, DMAP; (b) Ac_2O/DIEA; (c) 20% TFA/DCM, followed by 5% DIEA/DCM; (d) $H_2NCH_2CH_2CH_2NMe_2$; (e) **15**, DIPC, HOAt; (f) **16**, DIPC, HOAt; (g) concd. aq. NH_3.

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Table 1. Results of the UV-monitored thermal denaturation experiments.

Ligand	Ligand/duplex molar ratio	T_m [°C]	ΔT_m [°C][a]
1 (G-N^9-pyrr$_2$-Lys$_2$)	1:1	22.7	1.6
	2:1	23.6	2.5
2 (G-N^7-pyrr$_2$-Lys$_2$)	1:1	21.5	0.4
	2:1	21.5	0.4
3 (Ac-pyrr$_2$-Lys$_2$)	1.1	22.3	1.2
	2:1	23.3	2.2
4 (G-N^9-pyrr$_2$-NMe$_2$)	1:1	26.1	5.0
	2:1	27.9	6.8
5 (G-N^7-pyrr$_2$-NMe$_2$)	1:1	22.2	1.1
	2:1	22.7	1.6
6 (Ac-pyrr$_2$-NMe$_2$)	1:1	24.6	3.5
	2:1	26.0	4.9

[a] ΔT_m = the difference between the T_m of the [ligand – DNA complex] and the T_m of the duplex (21.1 °C) under the same conditions (2 μM duplex, 10 mM Na$_2$Pipes, pH = 7). Under the same conditions, the T_m of both the 1:1 and 2:1 netropsin–duplex complexes was 48.0 °C.

Several conclusions can be drawn from these data. The first is that the (dimethylamino)propyl tail favours the interaction of the ligands with the DNA duplex much more than the two lysines, as shown by the fact that the complexes formed by ligands **4**–**6** had higher T_m values than those involving ligands **1**–**3**. The larger size of the two lysines possibly disfavours the interaction with DNA and accounts for this effect.

None of the compounds studied was shown to destabilize the duplex. Some of the T_m values are very low, especially those of the complexes formed by ligand **2**, which suggests that either there is no interaction or that the binding affinity is very small. However, in other cases, the increase in the T_m values on changing the ligand/DNA ratio from 1:1 to 2:1 confirms that the interaction does indeed take place as a higher ligand concentration shifts the equilibrium and favours complex formation.[13]

Ligands containing the Ac-pyrr$_2$ moiety linked to a positively charged tail (**3** and **6**) did stabilize the duplex, as one would expect for dipyrrole-containing netropsin analogues. Note that ligand **6** [Ac-pyrr$_2$-NH(CH$_2$)$_3$NMe$_2$] has been reported to stabilize A,T-containing duplex DNA [poly-(dAdT)·poly(dAdT)],[12b] but to the best of our knowledge its effect on oligonucleotides with short A·T base-pair tracts flanked by G·C pairs has never been evaluated.

The effect of guanine can be assessed by comparing the behaviour of guanine-containing ligands with those acetylated at the N terminus, namely from the comparison of **1** and **2** with **3**, and **4** and **5** with **6**. The same behaviour was reproduced in the two series of ligands. On the one hand, ligands incorporating an N^7-alkylated guanine (**2** and **5**) were destabilizing with respect to those bearing the acetyl group (**3** and **6**, respectively). On the other, ligands bearing the N^9-alkylated guanine moiety (**1** and **4**) were clearly more stabilizing than the acetylated ones. As previously stated, these effects were more pronounced in ligands with the positively charged NMe$_2$ tail, ligand **4** showing the best stabilizing properties.

Finally, some preliminary CD experiments were carried out to assess the influence of guanine-pyrr$_2$-NMe$_2$ ligands on the duplex. For comparison purposes, the CD spectra of 1:1 and 2:1 ligand/duplex mixtures were recorded, in which the ligand was either netropsin, **4** or **5** (see Figure S1 of the Supporting Information). Although the effects were more pronounced for netropsin than for **4** or **5**, the main trends were the same in the three cases. All the spectra looked essentially like B-DNA, which suggests that the duplex remained basically unaltered upon binding the ligand. Moreover, a positive CD band (of higher intensity when the ligand/duplex ratio increased from 1:1 to 2:1) was observed at around 310 nm in all cases. This band is a typical feature of complexes formed between minor-groove binders and DNA,[23] and suggests that attachment of a guanine moiety does not interfere with the minor-groove binding properties of pyrr$_2$-NMe$_2$ ligands.

Conclusions

Solid-phase-based synthetic strategies have been devised for the preparation of dipyrrole-containing DNA ligands, which allowed six ligands differing in their N- and C-terminal ends to be prepared. All the ligands bore a positive charge at the C terminus, provided by the presence of either two lysines or a (dimethylamino)propyl group, and were either acetylated or linked to (guanin-9-yl)- or (guanin-7-yl)-acetyl moieties at the N terminus. Attachment of two lysine residues was shown not to be a good alternative to (dimethylamino)propyl groups for introducing positive charge into the ligand. With respect to the presence of guanine, interestingly, its effect was highly dependent on whether it was attached to the polyamide chain through either the N-7 or N-9 atom, the latter providing the highest duplex-stabilizing effect. The nature of the interaction between the (guanin-9-yl) group and dsDNA remains to be established. However, it seems clear that ligands incorporating this moiety show some promise as DNA minor-groove binders and deserve future studies, in particular with sequences containing short A/T tracts flanked by G·C pairs.

Experimental Section

General: Boc-L-Leu-OH, Boc-L-Lys(Fmoc)OH, *p*-methylbenzhydrylamine-polystyrene resin and Kaiser oxime resin were from Novabiochem. The 3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite derivatives of the 5′-*O*-DMT-2′-deoxynucleosides (A^{Bz}, C^{Bz}, G^{iBu} and T), the corresponding nucleoside-controlled pore glass supports and reagents and solvents for solid-phase oligonucleotide synthesis were from Glen Research. All products and solvents were reagent grade and were used without further purification except in the following cases: DMF was kept over molecular sieves (4 Å), and volatile amine contaminants were removed by bubbling N$_2$; anhydrous THF was obtained by distillation from Na lumps and benzophenone under N$_2$; anhydrous DCM was distilled from P$_2$O$_5$; and deionized water was filtered through a MilliQ (Millipore) system. Solid-phase oligonucleotide synthesis was carried out in an Applied Biosystems Expedite automatic synthesizer. Pyrrole-

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containing ligands were manually assembled in a polypropylene syringe fitted with a polyethylene filter disc. The following instruments were used to obtain spectroscopic data: Nicolet 510 FT-IR, Jasco V-550 with Peltier ETC-505T (UV and thermal denaturation experiments), Jasco J-810 spectropolarimeter fitted with a thermostatted cell holder (CD experiments), Varian Mercury 400 MHz (NMR), Bruker Digital Avance 600 MHz (NMR), HP-5988A (CI MS), VG-Quattro (ES MS), Perseptive Biosystems Voyager DE™-RP with a 337 nm N_2 laser (MALDI-TOF MS).

Synthesis of 4-Boc-amino-*N*-methylpyrrole-2-carboxylic Acid (10)

Methyl *N*-Methyl-4-nitropyrrole-2-carboxylate (8): Treatment of *N*-methylpyrrole (**7**) with trichloroacetyl chloride followed by reaction with nitric acid and then sodium methoxide to afford compound **8** was carried out as described in ref.[14] R_f (hexanes/ethyl acetate, 1:1, v/v) = 0.6. ^{1}H NMR ([D$_6$]DMSO, 200 MHz): δ = 8.26 (d, J = 1.8 Hz, 1 H, 5-H), 7.23 (d, J = 1.8 Hz, 1 H, 3-H), 3.89 (s, 3 H, NCH_3), 3.77 (s, 3 H, OCH_3) ppm. ^{13}C NMR ([D$_6$]DMSO, 50 MHz): δ = 159.8 (CO), 134 (C-5), 129.5 (C-4), 122.6 (C-2), 111.5 (C-3), 51.9 (OCH_3), 37.6 (NCH_3) ppm. IR (KBr): $\tilde{\nu}$ = 3149, 1717, 1542, 1420, 1318, 1195, 1115, 752 cm^{-1}. MS (CI, NH_3): m/z = 202 $[M + NH_4]^+$ (calcd. monoisotopic mass: 184.0).

Reduction of the Nitro Group and Isolation of 9: Compound **8** (500 mg, 1.72 mmol) was introduced into a round-bottomed flask and dissolved in a 6:1 mixture of anhydrous methanol/dichloromethane (12 mL). 10% Pd/C (165 mg) and ammonium formate (788 mg) were subsequently added, and the mixture was purged with Ar. The flask was gently heated to promote initiation of the reaction, which was identified by the formation of CO_2 bubbles. After 2.5 min, trifluoroacetic acid (1.25 mL, 16.8 mmol) was added. The reaction mixture was filtered to remove the solid catalyst. Elimination of the solvent under reduced pressure afforded 2.44 g of crude **9** mixed with salts, which was used without purification. MS (ES, positive mode): m/z = 155.2 $[M + H]^+$ (calcd. monoisotopic mass: 154.2).

Protection of the Amine and Ester Hydrolysis: Introduction of the Boc group was carried out essentially as described previously,[14] but by using 3 rather than 1.1 equiv. of di-*tert*-butyl dicarbonate. The resulting *N*-Boc-protected ester was characterized by NMR and MS. ^{1}H NMR ([D$_6$]DMSO, 200 MHz): δ = 9.10 (s, 1 H, NH), 7.08 (s, 1 H, 5-H), 6.59 (s, 1 H, 3-H) 3.76 (s, 3 H, NCH_3), 3.68 (s, 3 H, OCH_3), 1.34–1.41 (s, 9 H, Boc + small impurity of *tert*-butyl alcohol and Boc_2O) ppm. ^{13}C NMR ([D$_6$]DMSO, 50 MHz): δ = 161.4 (CO carboxylate), 153.4 (C=O Boc), 123.8 (C-2), 119.9 (C-4), 119.3 (C-3), 108.0 (C-5), 79.2 (C_q Boc), 51.5 (OCH_3), 36.7 (NCH_3), 28.8 (CH_3 Boc) ppm. MS (CI, NH_3): m/z = 255 $[M + H]^+$, 272 $[M + NH_4]^+$ (calcd. monoisotopic mass: 254.1). Finally, the *N*-Boc-protected pyrrole ester (1.42 g) was dissolved in methanol/water (3:1, v/v; 20 mL), and LiOH was added (1.17 g, 27.9 mmol). The mixture was heated at 45 °C, and the progress of the reaction was monitored by TLC (hexanes/ethyl acetate, 3:1, v/v). When the starting material could no longer be detected, methanol was eliminated under reduced pressure. The aqueous solution was poured into water/ethyl acetate (1:1, v/v; 30 mL), and H_2SO_4/water (1:1, v/v) was added with vigorous stirring, until the pH of the aqueous phase was 2–2.5. The two layers were separated, and the organic phase was dried with $MgSO_4$. Removal of the solvent under reduced pressure afforded 542 mg of pure **10** (**8** → **10** yield: 83%).

Characterization of 10: R_f (hexanes/ethyl acetate, 3:1, v/v) = 0.4; R_f (AcOEt) = 0.81. M.p. 157–160 °C (ref.[16] 160–161 °C). ^{1}H NMR ([D$_6$]DMSO, 200 MHz): δ = 12.1 (s, 1 H, COOH), 9.05 (s, 1 H, NH), 7.02 (s, 1 H, 5-H), 6.55 (s, 1 H, 3-H), 3.75 (s, 3 H, NCH_3), 1.41 (s, 9 H, Boc) ppm. ^{13}C NMR ([D$_6$]DMSO, 50 MHz): δ = 162.4 (COOH), 153.0 (C=O Boc), 123.4 (C-2), 120.2 (C-3), 119.3 (C-4), 108.0 (C-5), 79.0 (Cq Boc), 36.7 (NCH_3), 28.8 (CH_3 Boc) ppm. IR (KBr): $\tilde{\nu}$ = 3351, 2979, 1686, 1586, 1451, 1246, 1111 cm^{-1}. MS (ES, positive mode): m/z = 241.3 $[M + H]^+$ (calcd. monoisotopic mass: 240.3).

Synthesis of Guaninylacetic Acids 15 and 16

***N*^{2}-Acetylguanine (12):** Acetic anhydride (16.5 mL, 174.5 mmol) was added to a suspension of guanine (**11**; 10 g, 66.2 mmol) in *N*,*N*-dimethylacetamide, and the mixture was heated at 160 °C for 18 h. The resulting solution was cooled to room temperature, and a precipitate was formed, which was filtered and washed with absolute ethanol. The filtrate was concentrated under vacuum, which afforded more precipitate that was also filtered and washed. This procedure was repeated until no precipitate was formed upon concentration of the solution. All the solid material was combined to give 15.1 g of N^2,N^9-diacetylguanine [97% yield; MS (MALDI-TOF, sinapinic acid, negative mode): m/z = 233.9 (calcd. monoisotopic mass: 235.1)]. N^2,N^9-Diacetylguanine (2.99 g, 12.7 mmol) was suspended in ethanol/water (1:1, v/v; 15 mL), and the mixture was heated at reflux for 2 h. Upon cooling, a precipitate appeared. The mixture was concentrated to dryness, and the resulting solid was co-evaporated with acetonitrile (3×) to give 2.42 g of **12** (98% yield). ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 7.96 (s, 1 H, 8-H), 2.14 (s, 3 H, N^2-$COCH_3$) ppm. MS (MALDI-TOF, sinapinic acid, positive mode): m/z = 194.1 $[M + H]^+$, 216.1 $[M + Na]^+$, 232.0 $[M + K]^+$ (calcd. monoisotopic mass: 193.1).

Methyl (*N*^{2}-Acetylguanin-*x*-yl)acetate (*x* = 9: 13; *x* = 7: 14): DIEA (4.3 mL, 25.2 mmol) and methyl bromoacetate (1.3 mL, 13.7 mmol) were added to a suspension of **12** (2.42 g, 12.5 mmol) in DMF (36 mL) under Ar. After 20 h of stirring at room temperature, the solvent was removed in vacuo, and the residue was co-evaporated with methanol (3×). The resulting crude product was resuspended in methanol and added slowly and with vigorous stirring to water (95 mL) in an Erlenmeyer flask. The mixture was filtered under vacuum to afford **14** as a solid (1.35 g, 41% yield). The filtrate was concentrated under vacuum and chilled to yield a second solid product (**13**), which was also isolated by filtration under vacuum (0.96 g, 29% yield).

Methyl (*N*^{2}-Acetylguanin-9-yl)acetate (13): ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 12.05 (s, 1 H, N1-H), 11.69 (s, 1 H, N2-H), 7.95 (s, 1 H, 8-H), 5.01 (s, 2 H, CH_2COO), 3.69 (s, 3 H, $COOCH_3$), 2.15 (s, 3 H, $COCH_3$) ppm. ^{13}C NMR ([D$_6$]DMSO, 100 MHz): δ = 174.2 ($COCH_3$), 168.8 (COO), 155.5 (C-2), 149.7 (C-4), 148.7 (C-6), 140.9 (C-8), 120.4 (C-5), 53.2 ($COOCH_3$), 44.9 (CH_2COO), 24.4 ($NHCOCH_3$) ppm. [^{1}H-^{13}C] HMBC ([D$_6$]DMSO, 600 MHz): see Figure 2a. MS (MALDI-TOF, sinapinic acid, positive mode): m/z = 266.1 $[M + H]^+$, 288.1 $[M + Na]^+$, 304.1 $[M + K]^+$ (calcd. monoisotopic mass: 265.1).

Methyl (*N*^{2}-Acetylguanin-7-yl)acetate (14): ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 12.10 (s, 1 H, N1-H), 11.60 (s, 1 H, N2-H), 8.13 (s, 1 H, 8-H), 5.21 (s, 2 H, CH_2COO), 3.69 (s, 3 H, $COOCH_3$), 2.15 (s, 3 H, $NHCOCH_3$) ppm. ^{13}C NMR ([D$_6$]DMSO, 100 MHz): δ = 174.1 ($COCH_3$), 169.1 (COO), 157.6 (C-4), 153.3 (C-2), 147.8 (C-6), 145.6 (C-8), 112.4 (C-5), 53.2 ($COOCH_3$), 47.9 (CH_2COO), 24.4 ($NHCOCH_3$) ppm. [^{1}H-^{13}C] HMBC ([D$_6$]DMSO, 600 MHz): see Figure 2b. MS (MALDI-TOF, sinapinic acid, positive mode): m/z = 266.2 $[M + H]^+$, 288.2 $[M + Na]^+$, 304.1 $[M + K]^+$ (calcd. monoisotopic mass: 265.1).

(Guanin-*x*-yl)acetic Acid (*x* = 9: 15; *x* = 7: 16): Compound **13** (905 mg, 3.41 mmol) or **14** (900 mg, 3.39 mmol) was suspended in

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water/methanol/dioxane (2:1:4, v/v/v), and the pH was brought to 13 by adding 1 M sodium hydroxide. Immediately afterwards, 1 M hydrochloric acid was added until pH = 6 was attained. The organic solvents were removed in a rotary evaporator, and the resulting solution was acidified to pH = 2–2.5. The resulting solid was separated by vacuum filtration, washed with cold water and dried in a desiccator. This procedure afforded 656 mg of **15** (77% yield) and 834 mg of **16** (97% yield), respectively.

(Guanin-9-yl)acetic Acid (15): ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 12.00 (s, 1 H, N1-H), 8.10 (s, 1 H, 8-H), 5.04 (s, 2 H, CH_2COOH), 2.14 (s, 3 H, $NHCOCH_3$) ppm. ^{13}C NMR ([D$_6$]-DMSO, 100 MHz): δ = 173.2 (NHCO), 169.2 (COOH), 156.6 (C-4 and C-2), 146.8 (C-6), 144.9 (C-8), 111.7 (C-5), 47.5 (CH_2COOH), 23.6 ($NHCOCH_3$) ppm. MS (MALDI-TOF, sinapinic acid, positive mode): m/z = 252.1 [M + H]$^+$, 274.1 [M + Na]$^+$ (calcd. monoisotopic mass: 251.1).

(Guanin-7-yl)acetic Acid (16): ^{1}H NMR ([D$_6$]DMSO, 400 MHz): δ = 11.90 (s, 1 H, N1-H), 11.60 (s, 1 H, N2-H), 7.92 (s, 1 H, 8-H), 4.87 (s, 2 H, CH_2COOH), 2.14 (s, 3 H, $NHCOCH_3$) ppm. ^{13}C NMR ([D$_6$]DMSO, 100 MHz): δ = 173.4 (NHCO), 169.1 (COOH), 154.8 (C-2), 148.9 (C-4), 147.6 (C-6), 140.4 (C-8), 119.6 (C-5), 45.0 (CH_2COOH), 23.7 ($NHCOCH_3$) ppm. MS (MALDI-TOF, sinapinic acid, positive mode): m/z = 252.1 [M + H]$^+$, 274.1 [M + Na]$^+$ (calcd. monoisotopic mass: 251.1).

Synthesis of Pyrr$_2$-Lys$_2$ Ligands 1–3

Solid-Phase Assembly: *p*-Methylbenzhydrylamine-polystyrene resin (1.007 g, approx. 0.7 mmol/g) was thoroughly washed with DCM, 40% TFA/DCM and 5% ethyldiisopropylamine (DIEA) in DCM. After reaction with 0.5 equiv. of Fmoc-Leu-OH (Fmoc = 9-fluorenylmethoxycarbonyl) and DCC for 45 min, an aliquot was removed and the degree of substitution determined by quantification of the *N*-(9-fluorenylmethyl)piperidine formed after treatment with 20% piperidine in DMF, which showed it to be 0.24 mmol/g. Unreacted amine groups were blocked by acetylation (treatment with 10 equiv. of Ac_2O and ethyldiisopropylamine, 2 × 10 min). The Fmoc group was removed by treatment with 20% piperidine in DMF (3 + 10 min), and the bifunctional linker *N*-[9-(hydroxymethyl)-2-fluorenyl]succinamic acid[21] was incorporated by reaction with DCC (three-fold excess of the two reagents, 4 h). Attachment of the C-terminal lysine to the resin was achieved by reaction with DCC and DMAP [10-fold excess of Boc-L-Lys(Fmoc)-OH and DCC + 0.5 equiv. of DMAP, 90 min]. Possible unreacted hydroxy groups were acetylated (10 equiv. of Ac_2O and pyridine, 2 × 10 min). The amino group was deprotected by treatment with 40% TFA/DCM (5 + 25 min), and after the neutralization step (5% DIEA/DCM; 4 × 1 min) coupling of the second lysine was carried out (3 equiv. of amino acid and DCC, 90 min). The following steps were carried out twice to incorporate the two pyrrole units: (i) removal of the Boc group (40% TFA/DCM), (ii) neutralization of the protonated amines (5% DIEA/DCM) and (iii) reaction with Boc-pyrr-OH, DIPC and DMAP (molar ratio 5:5:2.5; 3 h). At this step, treatment of an aliquot with TFA, acetylation (pyrrole derivatives with free amino groups decompose into a mixture of products), reaction with concd. aq. ammonia/dioxane (1:1, v/v; 4 h) and analysis of the crude product confirmed that the pyrr-pyrr-Lys-Lys tetramer was attached to the resin. MS (MALDI-TOF, sinapinic acid, positive mode): m/z = 619.3 [M + H]$^+$, 641.3 [M + Na]$^+$, 657.2 [M + K]$^+$ (calcd. monoisotopic mass: 618.3). The resin was divided into three different batches, and deprotection of the N-terminal end (TFA, DIEA) was followed by either acetylation (Ac_2O/pyridine, 1:1, v/v; 10-fold excess; 2 × 10 min) or by incorporation of the desired guaninylacetic acid (**15** or **16**) by reaction with DIPC and HOAt (3 equiv. of each reagent, 3 h). Possible unreacted amino groups were capped (Ac_2O/pyridine, 1:1, v/v; 2 × 10 min).

Isolation and Characterization: Crude **3** was obtained after treatment of Ac-pyrr$_2$-Lys(Fmoc)$_2$-resin with 20% piperidine/DMF (3 × 20 min). The filtrate and washings (DMF) were collected, and the solvent was removed in vacuo. This was followed by suspension of crude **3** in water and the addition of diethyl ether (5 mL of each solvent), and separation of the organic phase and lyophilization. Ligand **3** was purified by reversed-phase medium-pressure liquid chromatography (gradient from 5 to 30% of B; A: H_2O/0.1% TFA; B: ACN/0.1% TFA, 600 mL of each solvent). Purity was assessed by reversed-phase HPLC (gradient from 5 to 35% of B in 30 min; A: H_2O/0.045% TFA; B: ACN/0.036% TFA, Kromasil C_{18}, 250 × 4 mm, 10 mm) and the structural identity confirmed by MALDI-TOF MS. Piperidine treatment of the (N^2-Ac-guaninylacetyl)-pyrr$_2$-Lys(Fmoc)$_2$ resins and washing of the resulting crudes with diethyl ether, as above, afforded the partially protected (N^2-Ac-guaninylacetyl)-pyrr$_2$-Lys$_2$ ligands. Deprotection of the guanine moiety was effected by reaction with concd. aq. ammonia at 55 °C for 15 h. Crude **1** and **2** were purified and characterized as described for **3**.

1: Overall yield: 18%. HPLC: t_R = 17.1 min. MS (MALDI-TOF, 2,5-dihydroxybenzoic acid, positive mode): m/z = 710.3 [M + H]$^+$, 732.2 [M + Na]$^+$, 748.2 [M + K]$^+$ (calcd. monoisotopic mass: 709.3).

2: Overall yield: 7%. HPLC: t_R = 17.1 min. MS (MALDI-TOF, 2,5-dihydroxybenzoic acid, positive mode): m/z = 710.3 [M + H]$^+$ (calcd. monoisotopic mass: 709.3).

3: Overall yield: 14%. HPLC: t_R = 18.1 min. MS (MALDI-TOF, 2,5-dihydroxybenzoic acid, positive mode): m/z = 561.4 [M + H]$^+$, 583.4 [M + Na]$^+$, 599.4 [M + K]$^+$ (calcd. monoisotopic mass: 560.3).

Synthesis of Pyrr$_2$-NMe$_2$ Ligands 4–6

Solid-Phase Assembly: Oxime Kaiser resin (300 mg, approx. 0.56 mmol/g) was washed with DCM and *N*-methylpyrrolidinone (NMP), and the first Boc-pyrr-OH unit was incorporated by reaction with DIPC and DMAP (5 equiv. of acid and DIPC, 2.5 equiv. of DMAP, 15 h). Unreacted hydroxy groups were blocked by acetylation (20 equiv. Ac_2O and 10 equiv. DIEA, 30 min). The amino group was deprotected by treatment with 20% TFA/DCM (3 × 0.5 min + 1 × 15 min) and neutralized (5% DIEA/DCM, 4 × 0.5 min). The second pyrrole building block (5 equiv.) was coupled (15 h) to the free amine activating the carboxy group with DIPC (5 equiv.) and DMAP (2.5 equiv.). Possible unreacted amino groups were capped (Ac_2O/DIEA, 2:1, v/v; 10-fold excess of Ac_2O; 30 min). The Boc-pyrr$_2$-oxime resin was divided into three batches. In one, the Boc group was eliminated (20% TFA), and the amine was acetylated (20 equiv. Ac_2O and 10 equiv. DIEA; 30 min). In the other two, removal of the Boc group was followed by incorporation of the corresponding guaninylacetic acid (activation with DIPC and HOAt, 10-fold excess of each reagent; 15 h). Possible unreacted amino groups were capped as indicated above.

Isolation and Characterization: Ac-pyrr$_2$-oxime resin was treated with a 1 M solution of *N*,*N*-dimethylpropane-1,3-diamine in anhydrous THF/DCM (1:1, v/v) at room temperature for 4 h. The combined filtrate and washings were concentrated to dryness, and crude **6** was purified by reversed-phase medium-pressure liquid chromatography, as indicated for the pyrr$_2$-Lys$_2$ ligands (gradient from 5 to 30% of B; A: H_2O/0.1% TFA; B: ACN/0.1% TFA, 600 mL of each solvent). The purity was assessed by reversed-phase HPLC (gradient from 5 to 35% of B in 30 min; A: H_2O/0.045%

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TFA; B: ACN/0.036% TFA), Kromasil C_{18} (250×4 mm, 10 µm). The structural identity was verified by MALDI-TOF MS and high-resolution ESI-MS for **4** and **5**. In the case of the (N^2-Ac-guaninylacetyl)-pyrr$_2$-oxime resins, treatment with *N*,*N*-dimethylpropane-1,3-diamine cleaved the oxime ester linkage and afforded the still protected (N^2-Ac-guaninylacetyl)-pyrr$_2$ ligands. Removal of the acetyl group was carried out by reaction with concd. aq. ammonia at 55 °C (3 h in the case of **4** and 6 h in the case of **5**). Crude **4** and **5** were purified and characterized as described for **6**.

4: Overall yield: 13%. t_R = 20.6 min. MS (MALDI-TOF, 2,5-dihydroxybenzoic acid, positive mode): *m*/*z* = 537.8 [M + H]$^+$ (calcd. monoisotopic mass: 537.3). HRMS (ESI, + mode): calcd. for $C_{24}H_{32}N_{11}O_4$ [M + H]$^+$ 538.2633; found 538.2624.

5: Overall yield: 7%. t_R = 20.7 min. MS (MALDI-TOF, 2,5-dihydroxybenzoic acid, positive mode): *m*/*z* = 537.9 [M + H]$^+$ (calcd. monoisotopic mass: 537.3). HRMS (ESI, + mode): calcd. for $C_{24}H_{32}N_{11}O_4$ [M + H]$^+$ 538.2633; found 538.2627.

6: Overall yield: 14%. t_R = 21.9 min. MS (MALDI-TOF, 2,5-dihydroxybenzoic acid, positive mode): *m*/*z* = 389.0 [M + H]$^+$ (calcd. monoisotopic mass: 388.2).

Preparation of Oligonucleotides: The oligonucleotides $^{5'}$GTGAATTCTG$^{3'}$ and $^{5'}$CAGAATTCAC$^{3'}$ were assembled on a 1 µmol scale by using the standard phosphite triester methodology. Deprotection was carried out with concd. aq. ammonia (15 h, 55 °C), and the two chains were purified by reversed-phase medium-pressure liquid chromatography (gradient from 5 to 30% of B; A: 0.05 M ammonium acetate; B: acetonitrile/water, 1:1, v/v; 600 mL of each solvent). The oligonucleotide purity was checked by reversed-phase HPLC (gradient from 5 to 35% of B in 30 min, same solvents as for purification, 1 mL/min, Kromasil C_{18}, 250×4 mm, 10 mm) and MALDI-TOF MS analysis was used for characterization.

$^{5'}$GTGAATTCTG^{3}: Overall yield: 49%. t_R = 14.7 min. MS (MALDI-TOF, 2,4,6-trihydroxyacetophenone and ammonium citrate, negative mode): *m*/*z* = 3055.9 [M – H]$^-$ (calcd. monoisotopic mass: 3056.5).

$^{5'}$CAGAATTCAC$^{3'}$: Overall yield: 35%. t_R = 13.9 min. MS (MALDI-TOF, 2,4,6-trihydroxyacetophenone and ammonium citrate, negative mode): *m*/*z* = 2993.4 [M – H]$^-$ (calcd. monoisotopic mass: 2994.6).

Thermal Denaturation Experiments: As neither of the compounds **1**–**6** proved to be stable for more than one week, even lyophilized and kept at –20 °C, UV-monitored thermal denaturation experiments were performed by mixing the DNA duplex with ligands freshly prepared (in other words, freshly deprotected and purified) and kept in the absence of light under Ar. Ligand purity was checked by HPLC immediately prior to use and rechecked after the thermal denaturation experiments. No degradation was detected in any case. Duplex solutions for the thermal denaturation studies were prepared by dissolving equimolar amounts of the two oligonucleotide strands in 10 mM Na$_2$Pipes, pH = 7 buffer. For each ligand two solutions were prepared, one with a concentration twice that of the other. The appropriate volumes of duplex and ligand solutions were mixed to obtain mixtures with the desired ligand/duplex ratio (1:1 or 2:1), and water was added to make the solutions either 2.0 µM in the two components or 2.0 µM in the duplex and 4.0 µM in the ligand. Annealing was carried out by heating at 60 °C for 15 min (75 °C when the effect of netropsin was evaluated) and allowing the samples to cool slowly to room temperature. The samples were then kept in a refrigerator (4 °C) or in an ice bath. Melting studies were carried out in quartz cells of 1 cm path length. Absorbance was monitored at 260 nm, and the samples were heated from 5 to 50 °C (or cooled from 50 to 5 °C) at a constant rate of 0.5 °C/min. A high nitrogen flow was used to purge the sample compartment to prevent water condensation at low temperatures. Mathematical analysis of the melting curves was carried out with the Microcal Origin software,[24] and T_m values were obtained from the first derivative. The error in the T_m data was estimated to be ±0.5 °C.

Supporting Information (see also the footnote on the first page of this article): Conditions and results of circular dichroism experiments.

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