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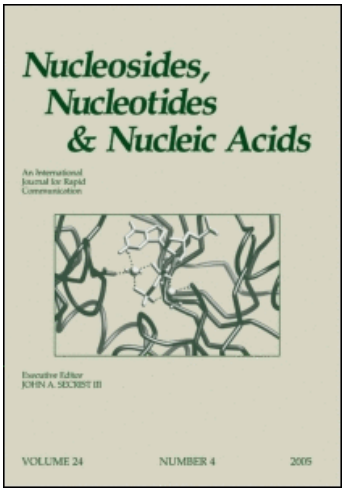

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BINDING OF A PORPHYRIN CONJUGATE OF HOECHST 33258 TO DNA. II. NMR SPECTROSCOPIC STUDIES DETECT MULTIPLE BINDING MODES TO A 12-MER NONSELF-COMPLEMENTARY DUPLEX DNA

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BINDING OF A PORPHYRIN CONJUGATE OF HOECHST 33258 TO DNA. II. NMR SPECTROSCOPIC STUDIES DETECT MULTIPLE BINDING MODES TO A 12-MER NONSELF-COMPLEMENTARY DUPLEX DNA

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ABSTRACT

We have probed by ^{1}H NMR spectroscopy the molecular basis of the interaction between Hoechst 33258 conjugated to a *des*-metalloporphyrin and a non self-complementary duplex DNA sequence, designed on the known chemical nuclease selectivity of this system. The imino NMR spectra are consistent with two distinct families of structure, that is, PORHOE binding either way along the duplex. 2D spectral, T_2, and linewidth data suggest multiple species within the two conformational families.

Key Words: DNA-binding; Minor groove; Ligand; NMR; NOESY; DNA duplex.

INTRODUCTION

Inhibition of gene expression by creating specific irreversible damage to DNA is a potentially useful strategy in the development of novel molecular biological tools and, in the longer term, of new therapeutic agents. Such a goal depends on the ability to design and synthesize molecules able to damage selected sequences in the

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targeted DNA. A molecular conjugate associating a tris (4-*N*-methylpyridiniumyl) Mn(III) porphyrin motif to Hoechst is a chemical nuclease (1,2), causing cleavage/damage to two different areas of a selected 35-mer ds oligonucleotide. This could indicate two possible orientations of the molecule with respect to the cleavage sites, or there may be a sequence-induced configurational effect on the local minor groove creating "hot sites" with respect to cleavage susceptibility. From physicochemical considerations a model was proposed for the interaction of the Mn(III) porphyrin: Hoechst conjugate with poly (dA) · (dT), poly [d(A–T) · d(A–T)], and poly (dG) · $(dC)^2$.

The investigation of the interaction of the nonmetallated porphyrin conjugate molecule H_2TrisMPyP-Hoechst 33258 (PORHOE, Scheme 1) with a non self-complementary, 12-mer double-stranded oligonucleotide by UV–vis spectrophotometry and melting temperature analysis indicated more than one class of strong binding site (see Part I of this paper).

We report here a detailed high-field ^{1}H NMR study of the *des*-metallo-PORHOE conjugate with the duplex, d(CGAATTGTATGC)·d(GCATACAATTCG). This 12-mer oligonucleotide was designed to contain the affinity sites of both Hoechst 33258 (5′-AATT-3′) and MnTMPyP (5′-TAT-3′) based on the nuclease experiments for the 35-mer (2). Because of the paramagnetism of the Mn(III) nucleus, we first studied the interaction of the non-metallated derivative with the oligonucleotide.

MATERIALS AND METHODS

The atom labelling of the duplex from of d(CGAATTGTATGC) · d(GCATACAATTCG) used in this study is shown in Scheme 1. The conjugate porphyrin–Hoechst 33258 (PORHOE) was synthesized and characterized as previously reported (3,4). Hoechst 33258 was from Aldrich Chemical Co. The 12-mer oligonucleotides CGAATTGTATGC and GCATACAATTCG were purchased from the Oswell DNA unit (University of Southampton).

An equimolar mixture of the two strands was obtained by scaling the amount of each oligonucleotide relative to a standard amount of internal NMR calibrant (TSP). Thus, an aliquot of TSP (4 μL of 0.0295 *M* in D_2O) was added to 600 μL NMR solution of each oligonucleotide in D_2O. The amount of oligonucleotide was calculated from the 1D ^{1}H NMR spectrum relative to the integral area of the TSP ^{1}H signal. The DNA duplex (final duplex concentration 2.5 m*M*) was prepared by dissolving 1.5 μmol of each oligonucleotide strand in 0.6 mL of NMR buffer containing 10 m*M* NaH_2PO_4/Na_2PO_4 pH 7.20, 100 m*M* NaCl, 0.1 m*M* EDTA and 0.12 m*M* TSP, prepared in 99.96% D_2O. This duplex was lyophilized three times against 99.96% D_2O to reduce the water signal. A 12-mer–PORHOE complex (1:1) was produced by stepwise addition of aliquots of PORHOE (1.5 μmol in five aliquots) prepared in D_2O to the 0.6 mL of 2.5 m*M* DNA solution dissolved in the NMR buffer. The 1:1 molar ratio in the 12-mer–PORHOE complex was confirmed

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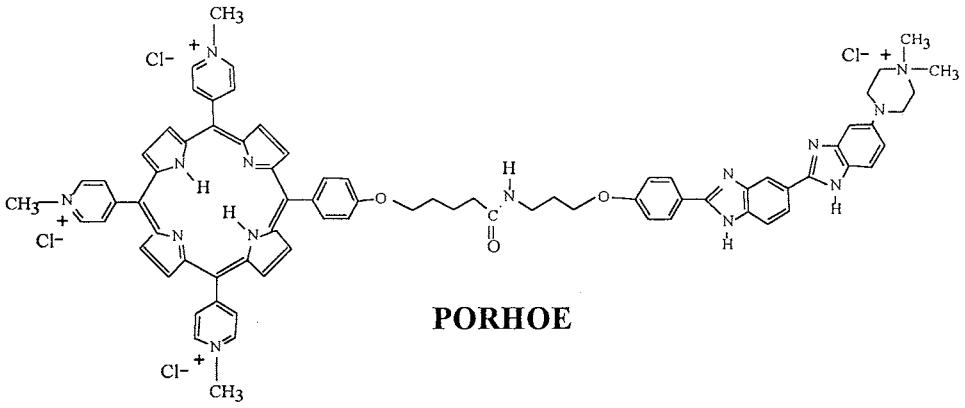

PORHOE

5′ 1C 2G 3A 4A 5T 6T 7G 8T 9A 10T 11G 12C 3′

3′ 24G 23C 22T 21T 20A 19A 18C 17A 16T 15A 14C 13G 5′

Scheme 1. Structure of the cationic conjugate porphyrin–Hoechst 33258 (PORHOE) and sequence and numbering system for the 12-mer DNA duplex.

by the UV–vis spectrophotometry using the following molar extinction coefficients: $\varepsilon_{340\ \mathrm{nm}} = 40{,}000\ M^{-1}\ \mathrm{cm}^{-1}$ and $\varepsilon_{430\ \mathrm{nm}} = 75{,}000\ M^{-1}\ \mathrm{cm}^{-1}$. To perform REFOPT and REFOPTNY experiments in H_2O media, samples containing 12-mer duplex or 12-mer–PORHOE complex were lyophilized and re-dissolved in the H_2O/D_2O mixture (9:1, v/v).

NMR data were recorded for free oligonucleotide chains, free 12-mer duplex, and 12-mer–PORHOE complex (1:1) using a dual (inverse) $^1H(X)$ probe-head on a Varian Unity 400 NMR spectrometer (operating at 400.130 MHz) equipped with a Sun Sparc I host computer running VNMR system software version 4.3.

1D ^1H NMR spectra in D_2O media were acquired at 30°C for free duplex and 12 and 30°C for the PORHOE complex. Data were collected into 16K data points over a spectral width of 5 kHz; 128 transients were acquired per spectrum with a 1.5-s recycle delay, during which the residual *H*OD resonance was suppressed by continuous low-power irradiation.

Pure absorption 2D TOCSY (5) and NOESY (6–8) data were collected with quadrature detection into 4096 complex data points for $2 \times 512\ t_1$ increments in hypercomplex phase-sensitive mode (9); 32 transients were acquired per t_1 increment. The residual *H*OD resonance was suppressed by continuous, low-power irradiation during the 1.5-s recycle time. Data were acquired over a 5 kHz spectral width (final F_2 digital resolution of 1.22 Hz/pt.). NOESY data were acquired with mixing times of 50, 100, 200, 250, 300, and 500 ms at 12 and 30°C. TOCSY data were acquired with a mixing time of 55 ms at 12 and 30°C. All 2D data sets were acquired nonspinning in an interleaved fashion to reduce t_1 noise and other artifacts.

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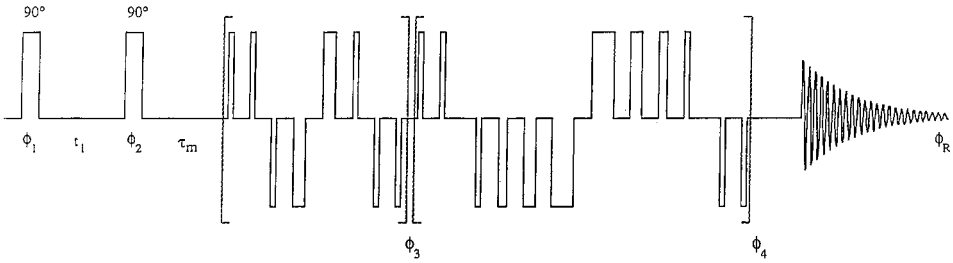
T_2 measurements used the Carr–Purcell–Meiboom–Gill (CPMGT2) pulse sequence at 12°C. Data were collected into 16K data points over a 5 kHz spectral width. For each spectrum, 384 transients were acquired (8.16 s relaxation delay, 2 ms interpulse delay, echo times from 4 to 194 ms). The *H*OD resonance was not suppressed.

REFOPTNY data, accumulated for the complex in H_2O/D_2O (9:1), were collected with quadrature detection into 4096 complex data points for $2 \times 256\, t_1$ increments in hypercomplex phase-sensitive mode (9); 96 and 240 transients were acquired for each free induction decay, for free duplex, and for the PORHOE complex. The REFOPTNY pulse sequence (Scheme 2) replaces the third pulse of a conventional NOESY sequence with the REFOPT sequence (10). REFOPT consists of a selective 90° pulse sequence followed by a selective 180° sequence; both sequence elements contain equal number 0° and 180° pulse-shifted pulses, with flip angles numerically optimized to give a null effect both on and close to resonance, but approximately 90° and 180° rotations respectively for the remainder of the spectrum of interest. The overall effect is to give full excitation of the signals of interest but efficient suppression of water signals; signal phases are constant except for a 180° discontinuity at the transmitter frequency. The REFOPTNY pulse sequence is particularly useful on spectrometers not equipped with pulsed field



Scheme 2. Pulse sequence for the REFOPT experiment. The initial 90° pulse, evolution period t_1, second 90° pulse and mixing period τ_m from the first part of a normal NOESY sequence, but the usual 90° NOESY read pulse is replaced by a sequence consisting of an 8-pulse 90° net flip angle segment followed by a 12-pulse 180° segment. The flip angles of the individual pulses and the lengths of the delays between them are optimized to give flat excitation over most of the spectrum of interest, but zero excitation both at and close to the water signal. The 90° pulse segment has the form 7.09° – 0.183 τ – 5.26° – 0.158 τ – 7.61° – 0.198 τ – 53.75° – 0.191 τ – 53.75° – 0.198 τ – 7.61° – 0.258 τ – 5.26° – 0.183 τ – 7.09°, and the 180° segment 5.53° – 0.174 τ – 6.03° – 0.371 τ – 11.70° – 0.183 τ – 34.26° – 0.182 τ – 72.46° – 0.172 τ – 138. 57° – 0.163 τ – 138.57° – 0.172 τ – 72.46° – 0.182 τ – 34.26° – 0.183 τ – 11.70° – 0.371 τ – 6.03° – 0.174 τ – 5.53° – 0.635 τ, where the overbars indicate 180° phase shifts and the delay τ, here 700 μs, controls the excitation bandwidth. The short delay between the end of the 90° segment and the start of the 180° segment, here 10 μs, allows for the change in radiofrequency phase. An additional delay of 162 μs, determined by the finite 90° pulse width of 27 μs, was added between the end of the 180° segment and the onset of data acquisition to minimize frequency-dependent phase errors. The phase cycling follows the pattern ϕ = (0000 2222), $\phi_2 = 0$, ϕ_3 = (3210 3210 0321 0321 1032 1032 2103 2103), ϕ_4 = (0000 0000 1111 1111 2222 2222 3333 3333), ϕ_R = (0123 2301 1230 3012 2301 0123 3012 1230), with all phases incremented by 1 unit every 32 transitions. Phase shifts are indicated in units of 90°.

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gradients. A 1.5-s recycle delay and 175 ms mixing were used in the NOESY portion of the sequence. Data were acquired over a 14 kHz spectral width in both frequency dimensions.

The 1D REFOPT pulse sequence (10) was used in variable temperatures experiments to monitor the oligonucleotide imino proton resonance region. The temperatures used in VT experiments were 11°, 15°, 20°, 25°, 30°, 35°, 40°, 45°, 50°, 52°, 54°, 56°, and 58°C for 12-mer duplex, and 12°, 15°, 20°, 25°, 30°, 35°, 40°, 45°, 50°, 55°, 60° and 70°C for 12-mer–PORHOE complex. Data were collected into 32K data points (spectral width of 10 kHz); 60 transients were accumulated with a 2-s recycle delay.

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RESULTS

Assignment of nonexchangeable protons of free 12-mer duplex. Free duplex proton resonances were assigned by combined use of TOCSY and NOESY data. Cytosine H_5/H_6 and thymidine CH_3/H_6 connectivities were unambiguously attributed by means of the 2D-TOCSY spectrum. Figure 1 shows the full ^{1}H NOESY spectrum of d(CGAATTGTATGC) · d(GCATACAATTCG) duplex at 30°C with regions **A** and **B** showing H6/H8–H1′/H3′/H5 and H6/H8–H2′/H2″/CH_3 connectivities, respectively. Qualitative analysis of NOESY spectra suggested the free 12-mer duplex to have the right-handed DNA structure. Thus, nonexchangeable ^{1}H resonances of the duplex were identified by the classical "interaction walk" for right-handed DNA, in which aromatic H6/H8/H5/CH_3 protons give through-space connectivities with the H1′/H2′/H2″ sugar ring protons of the same nucleotide

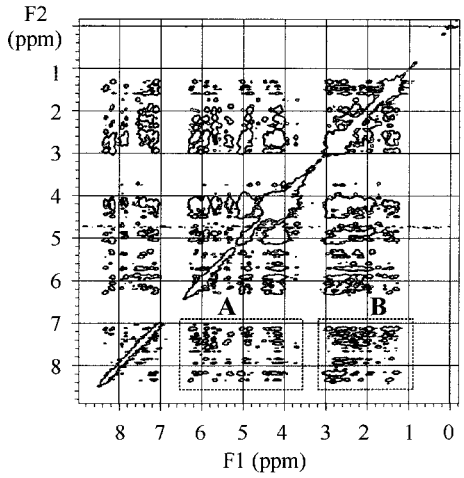

Figure 1. ^{1}H NOESY spectrum (400 MHz) of 12-mer duplex d(CGAATTGTATGC) · d(GCATACAATTCG) recorded at 30°C in 10 m*M* sodium phosphate buffer, pH 7.0, containing 100 m*M* NaCI and 0.1 m*M* EDTA in 99.96% D_2O. The region **A** represents H6/H8–H1′/H3′/H5 connectivities, the region **B** shows H6/H8–H2′/H2″/CH_3 connectivities.

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residue, and also with those of the 5′-adjacent nucleotide residue (11). Assignments for d(CGAATTGTATGC) and d(GCATACAATTCG) are in Table 1.

Imino proton assignment. Initial assignment of exchangeable imino protons was effected using variable temperature 1D REFOPT from 10°C to 60°C, with 5°C increments up to 50°C and 2°C increments up to 60°C. Imino proton identification was based on the assumption that a duplex melts from the ends inward, resulting in initial line-broadening of the imino proton signals of the terminal base pairs, followed by sequential broadening for more internal base pairs. The last signals to broaden are those from the central base pairs. The initial assignment was confirmed by a 2D-REFOPTNY experiment (500 MHz, 11°C), based on the connectivities between the aromatic protons (already assigned from the TOCSY and NOESY spectra) and amino proton of the same base pair with imino protons of the neighboring base pairs (Fig. 2)

$$\ldots \mathrm{N}_{i-1}(\mathrm{H6/H8/H5}) \leftrightarrows \mathrm{N}_{i-1}(\mathrm{G(N1H)/T(N3H)} \leftrightarrows \mathrm{N}_{i}(\mathrm{G(N1H)/T(N3H)}) \ldots$$

with N as any nucleotide residue (assignments in Table 2). We were unable to differentiate end base-pairs (^{1}C−^{13}G and ^{12}C−^{24}G) imino protons due to signal overlap.

ID NMR analysis of 12-mer–PORHOE complex in D_2O and in H_2O solutions. Complex formation (1:1) between PORHOE and 12-mer duplex was monitored by ID ^{1}H NMR titration experiments both in D_2O and H_2O solution. For the 12-mer–PORHOE complex, the duplication of some imino proton signals from several base pairs (Table 2) marked the suspected minor groove location limits of

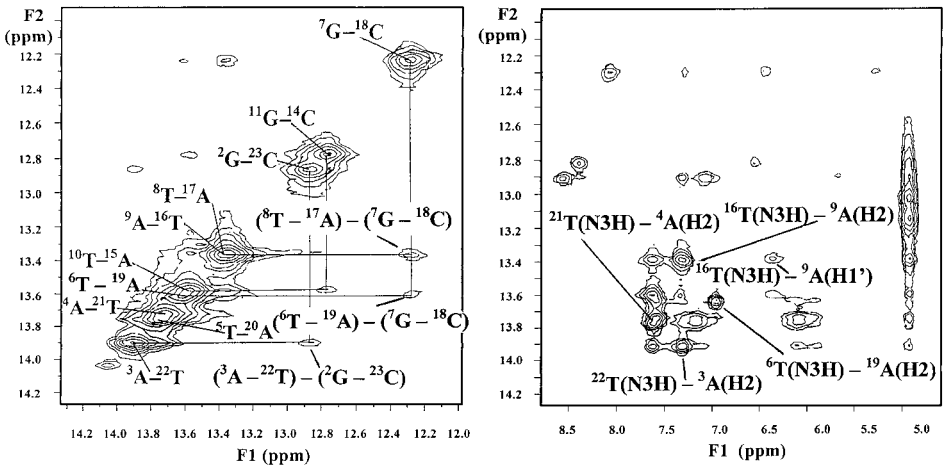

Figure 2. Expanded regions of the REFOPTNY spectrum (500 MHz) of 12-mer duplex d(CGAATTGTATGC) · d(GCATACAATTCG) containing imino–imino and imino–aromatic proton areas. Spectrum was recorded at 11°C in 10 m*M* sodium phosphate buffer, pH 7.0, containing 100 m*M* Nacl and 0.1 m*M* EDTA in H_2O/D_2O (90%/10%). Through-space connectivities between imino protons of the neighboring nucleotide bases are shown, along with connectivities between imino protons and respective aromatic protons.

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Table 1. Resonance Assignments[a] for Nonexchangeable Protons of Free Duplex d(CGAATTGTATGC) · d(GCATACAATTCG) (first row entries) and of the Complex PORHOE · d(CGAATTGTATGC) · d(GCATACAATTCG) (second row entries, in bold)

	CH_3	H5	H6	H8	H1′	H2′	H2″	H3′	H4′	H5′	H5″
Assignments for d(^{1}C^{2}G^{3}A^{4}A^{5}T^{6}T^{7}G^{8}T^{9}A^{10}T^{11}G^{12}C)											
^{1}C		5.83	7.56		5.66	1.72	2.28	A	4.65	[b]	[b]
	–	**5.81**	**7.54**	–	[b]	**1.72**	**2.19**	[b]	[b]	[b]	[b]
^{2}G				7.92	5.34	2.65	2.75	4.98	4.66	4.28	4.05
	–	–	–	**7.91**	[b]	**2.68**	[b]	[b]	[b]	[b]	[b]
^{3}A				8.18	6.03	2.75	2.93	5.09	4.48	4.26	4.26
	–	–	–	**8.22**	**6.16**	[c]	[c]	**5.05**	[c]	[c]	[c]
^{4}A				8.14	6.17	[c]	[c]	5.01	4.48	[c]	[c]
	–	–	–	**8.18**	**6.19**	[c]	[c]	[c]	[c]	[c]	[c]
^{5}T	1.28		7.10		5.85	2.57	2.61	5.02	4.84	4.30	4.16
	1.30	–	**7.14**	–	**5.89**	**2.34**	**2.60**	[b]	**4.85**	**4.25**	**4.12**
^{6}T	1.56		7.28		5.84	2.17	2.51	4.90	4.83	4.18	4.13
	1.59	–	**7.29**	–	**5.89**	**2.18**	**2.55**	[b]	**4.43**	**4.18**	**4.09**
^{7}G				7.83	5.89	2.61	2.74	4.96	4.91	4.37	4.16
	–	–	–	**7.84**	**5.91**	**2.57**	[b]	[b]	[b]	[b]	[b]
^{8}T	1.40		7.22		5.68	2.13	2.48	4.94	4.88	4.21	4.16
	1.41	–	**7.25**	–	**5.66**	[c]	[c]	**5.01**	**4.81**	[c]	[c]
^{9}A				8.30	6.24	2.66	2.90	5.02	4.41	4.18	4.18
	–	–	–	**8.34**	**6.26**	**2.75**	**2.92**	**5.13**	**4.43**	**4.25**	**4.12**
^{10}T	1.42		7.11		5.69	1.95	2.33	5.02	4.84	4.30	4.16
	1.40	–	7.11	–	**5.69**	**2.01**	**2.33**	**5.29**	**4.85**	[b]	**4.16**
^{11}G				7.85	5.95	2.53	2.68	4.96	4.86	4.37	4.16
	–	–	–	**7.87**	[b]	[b]	**2.61**	[b]	[b]	[b]	[b]
^{12}C		5.33	7.40		5.70	1.97	2.17	4.95	4.49	4.19	4.07
	–	**5.32**	**7.38**	–	**6.14**	[b]	**2.16**	[b]	**4.49**	[c]	[c]
Assignments for d(^{13}G^{14}C^{15}A^{16}T^{17}A^{18}C^{19}A^{20}A^{21}T^{22}T^{23}C^{24}G)											
^{13}G				7.93	5.96	2.60	2.76	4.85	4.70	4.22	4.11
	–	–	–	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]
^{14}C		5.42	7.47		5.68	2.14	2.47	4.88	4.88	4.20	4.14
	–	**5.39**	**7.46**	–	**5.67**	**2.20**	**2.45**	[b]	[b]	**4.23**	**4.06**
^{15}A				8.33	6.27	2.70	2.92	5.03	4.43	4.17	4.17
	–	–	–	**8.36**	**6.27**	**2.75**	**2.93**	**5.06**	**4.51**	**4.21**	[b]
^{16}T	1.48		7.15		5.57	1.95	2.33	5.03	4.85	4.26	4.14
	1.47	–	**7.18**	–	**5.53**	**1.98**	**2.35**	**5.04**	**4.86**	**4.23**	**4.13**
^{17}A				8.20	6.12	2.62	2.79	5.01	4.39	4.14	4.14
	–	–	–	**8.22**	**6.16**	**2.70**	**2.91**	**5.05**	[c]	[c]	[c]
^{18}C		5.29	7.24		5.33	1.88	2.24	4.78	[b]	4.13	4.11
	–	**5.29**	**7.28**	–	**5.29**	**2.03**	**2.17**	**4.80**	[b]	**4.17**	**4.11**
^{19}A				8.15	5.90	[c]	[c]	5.01	4.48	[c]	[c]
	–	–	–	**8.18**	**5.96**	[c]	[c]	**5.05**	4.48	[c]	[c]
^{20}A				8.15	6.15	[c]	[c]	5.01	4.48	[c]	[c]
	–	–	–	**8.15**	**6.19**	[c]	[c]	**5.05**	[c]	[c]	[c]
^{21}T	1.31		7.13		5.87	2.57	2.92	5.03	4.82	4.28	4.15
	1.37	–	**7.18**	–	[b]	[b]	[b]	**5.04**	**4.86**	**4.23**	**4.13**
^{22}T	1.57		7.39		6.09	1.97	2.17	4.89	4.84	4.19	4.07
	1.59	–	**7.40**	–	**6.14**	**1.98**	**2.17**	[b]	[b]	[c]	[c]
^{23}C	5.70		7.49		5.74	2.03	2.37	4.88	4.88	4.17	4.14
	–	**5.68**	**7.48**	–	**5.74**	[c]		[b]	[b]	**4.31**	**4.17**
^{24}G				7.93	6.14	2.60	2.76	4.85	4.70	4.22	4.11
	–	–	–	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]

[a]Based on ^{1}H NOESY spectra (400 MHz) recorded at 30°C in 10 m*M* phosphate buffer, pH 7.0, 10 m*M* NaCl, and 0.1 m*M* EDTA in D_2O.
[b]Signals were not detected.
[c]Signals are crowded.

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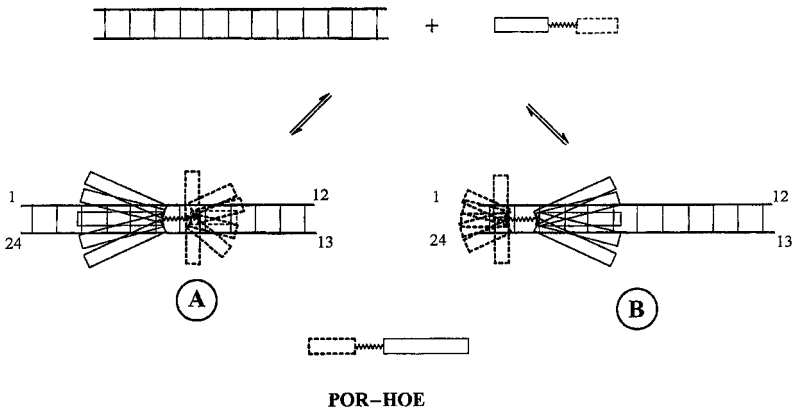
Table 2. Resonance Assignments[a] for Imino Protons of Free Duplex d(CGAATTGTATGC) · d(GCATACAATTCG) (middle column) and of the Complex PORHOE · d(CGAATTGTATGC) · d(GCATACAATTCG) (right column)

	Imino Proton Signals	
Base Pairs	Free 12-mer Duplex	PORHOE–12-mer Duplex
^{1}C–^{24}G	13.10 or 12.98	–
^{2}G–^{23}C	12.85	12.84
^{3}A–^{22}T	13.89	13.82 and 13.78
^{4}A–^{21}T	13.74	13.68
^{5}T–^{20}A	13.70	13.66
^{6}T–^{19}A	13.60	13.57
^{7}G–^{18}C	12.26	12.35 and 12.27
^{8}T–^{17}A	13.34	13.30 and/or 13.24 and/or 13.19 and/or 13.14
^{9}A–^{16}T	13.34	13.30 and/or 13.24 and/or 13.19 and/or 13.14
^{10}T–^{15}A	13.57	13.51
^{11}G–^{14}C	12.76	12.73
^{12}C–^{13}G	13.10 or 12.98	–

[a]Recorded at 11°C in 10 m*M* phosphate buffer, pH 7.0, 10 m*M* NaCl, 0.1 m*M* EDTA, prepared in 90% H_2O/10% D_2O.

the Hoechst (12,13). The signals of the imino protons of ^{7}G–^{18}C and ^{3}A–^{22}T base pairs each become resolved into two signals of equal intensity as 1:1 molar ratio approached. The imino ^{1}H protons of the ^{8}T–^{17}A and ^{9}A–^{16}T base pairs, which overlapped in the spectrum of free duplex, split into four signals. The signal duplication may indicate the simultaneous presence of two main conformational families A and B originating from two possible alignments of Hoechst-moiety within the DNA minor groove (Scheme 3). Exchange between them is slow on the NMR



Scheme 3. Representation of possible forms of the PORHOE-12-mer duplex complex. The bisbenzimidazole region is bound in both orientations (A or B) with respect to the AATT tract with the cationic *N*,*N*-dimethylpiperazino nitrogen atom directed toward either the 1, 24 (Case A) or the 12, 13 (Case B) terminus. In case A, the porphyrin-binding region is located approximately at ^{7}G–^{18}C and in case B at approximately the ^{1}C–^{24}G/^{2}G–^{23}C region.

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time-scale during denaturation–renaturation. Almost all imino proton signals of PORHOE · 12-mer complex shift to the high-field area by 0.01–0.07 ppm (Table 2) as a result of shielding effect from PORHOE. In addition, new imino proton signals appeared at 11.2, 11.35, 11.45, 12.5, 12.6, and 14.05 ppm, probably due to the formation of new hydrogen bonds formed by benzimidazole ring protons of the Hoechst moiety and oligonucleotide protons.

Another remarkable feature of the ^{1}H NMR spectrum of the 12-mer:PORHOE complex was very strong signal broadening (observed both for oligonucleotide and for PORHOE resonances) on complex formation. Figure 3 shows selected imino, aromatic, and H2′/H2″/CH_3 proton regions of the 12-mer–PORHOE complex (1:1) in comparison with the respective resonance regions of the free 12-mer duplex. The range of line half-widths changes from 4–6 Hz for free duplex to 23–25 Hz for the 12-mer–PORHOE complex. One possible source of such extensive line broadening is the existence of multiple duplex forms within each conformational family A and B, originating from different locations of the POR moiety on the duplex surface due to the absence of specific interactions. An additional contribution to line broadening could arise from exchange processes between the above duplex forms. To investigate the contribution of dynamic factors to the line broadening, T_2 values for the 1:1 oligo-PORHOE complex with the CPMGT2 pulse sequence were measured. These were found to be in the range 30–80 ms, giving a range of 10 to 4 Hz for the natural line-widths, compared to line-widths measured directly

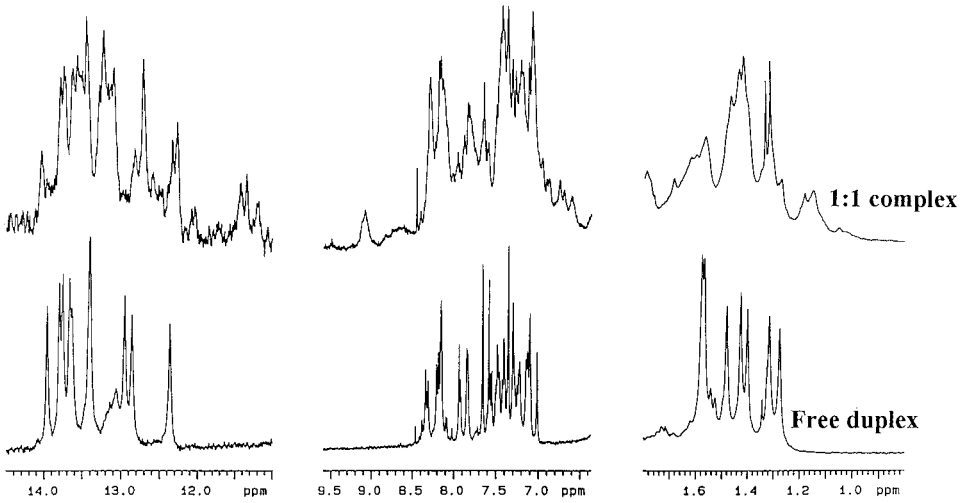

Figure 3. Comparison of three spectral regions (CH_3 groups of thymine bases, aromatic ^{1}H protons, and imino ^{1}H protons) of the 1D spectra of the free duplex (bottom) and of the 1:1 complex formed between PORHOE and d(CGAATTGTATGC)-d(GCATACAATTCG). Data for nonexchangeable protons (1.0–8.0 ppm) were obtained by standard 1D ^{1}H NMR experiments and acquired in 10 m*M* sodium phosphate buffer, pH 7.0, containing 100 m*M* NaCl and 0.1 m*M* EDTA in 99.8% D_2O at 30°C. Data for exchangeable imino protons (12.00–14.00 ppm) were obtained in the same buffer prepared in 90% H_2O/10% D_2O, at 30°C using the 1D-REFOPT sequence.

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from ID NMR spectra, which were of the order of 25 Hz. The relatively long T_2 values measured imply that any exchange between conformations is slow on the time-scale (2 ms) of the refocusing, ruling out more rapid processes as the line broadening.

2D NMR analysis of 12-mer–PORHOE duplex. The analysis of the 12-mer–PORHOE complex by 2D NMR spectroscopy showed only very weak cross-peaks, all at the same frequencies as observed in TOCSY or NOESY spectra of the free 12-mer duplex (Table 1, in bold). These cross-peaks correspond to the bound form of 12-mer duplex, present to a small extent in the sample. No cross-peaks from the bound form of 12-mer–PORHOE could be detected, presumably because the multiple conformations of the system give rise to large numbers of very weak cross-peaks.

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DISCUSSION

The 1D REFOPT data for PORHOE:duplex (1:1) showing the shift of the most imino protons to high field and the appearance of new imino protons signals, as well as the splitting of signals from ^{7}G$-^{18}$C, ^{3}A$-^{22}$T, ^{8}T$-^{17}$A, and ^{9}A$-^{16}$T base pairs, indicate interaction of PORHOE with duplex oligo. The doubling of imino signals may indicate the existence of two main conformational families (Scheme 3). This is consistent with spectrophotometric data for PORHOE with this duplex (see Part I of this paper), minimally being explained by two independent binding processes, a simple noncooperative process (apparent dissociation constant for complex $\sim 0.2\,\mu M$) and a cooperative binding mode (apparent dissociation constant $\sim 1\,\mu M$ and Hill coefficient ~ 2).

The strong signal broadening observed in ^{1}H NMR spectra for both exchangeable and nonexchangeable protons strongly suggests the presence of multiple duplex forms within each conformational family, originating from random distribution of the POR-moiety on the duplex surface, perhaps, due to nonspecific electrostatic interactions. The ability of cationic porphyrins to provider nonspecific electrostatic interactions with both ds and ssDNA was clearly demonstrated in the UV–vis experiments on competitive binding in the presence or absence of NaCl (see Parts I and III of this paper). Any exchange between the above multiple duplex forms is slow on the NMR time-scale based on T_2 measurements, as the range of $\Delta\nu_{1/2}$ (20–25 Hz) significantly exceeded natural line half-widths (4–10 Hz) evaluated from T_2 magnitudes. Thus, in addition to the evidence of specific and strong complexation of PORHOE to the 12-mer duplex suggested by direct cleavage studies (2), T_m studies, and UV–vis spectrophotometry (see Part I of this paper), the NMR data show the presence of two main conformational families with multiple forms within each.

The interaction of PORHOE with dsDNA is likely to proceed predominantly due to binding of the Hoechst moiety within the DNA minor groove, accompanied by nonspecific electrostatic interaction between the positively charged POR-part and the negatively charged DNA phosphate groups. In this case the Hoechst moiety

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serves as a "shape anchor" for PORHOE, providing binding in the vicinity of AATT-tracts. After thermal denaturation of the duplex part, accompanied by the release of the Hoechst moiety from the DNA minor groove, the POR-part starts to serve as an "electrostatic anchor" in the PORHOE interaction with the separated single-stranded DNA chains.

A physical model of the binding process for PORHOE may be as follows. The Hoechst portion initially, to guide the location of PORHOE by its *external* recognition of AATT from the minor groove. This needs little DNA distortion and locates the porphyrin approximately correctly. It is now easier (than for a free porphyrin with no Hoechst moiety) to intercalate as the intercalation process has become an entropically favored "intramolecular reaction," the "chelate effect." Once the porphyrin site is intercalated, the nearby duplex partially unwinds and distorts, weakening the net binding strength of the Hoechst moiety relative to a free Hoechst ligand (with no porphyrin attached).

Thus, the system overall consists of a homing device, which is essentially a strong, external binding recognition of a target on DNA (provided here by the Hoechst moiety), and in turn allows a new binding interaction at a remote site for another part of the ligand assembly, that is, for the porphyrin. Binding of the porphyrin leads to major structural alterations of the oligonucleotide duplex, but is stronger than expected for an isolated porphyrin with no Hoechst moiety. The binding of the porphyrin is tightened by the entropy-surmounting action of the homing device (Hoechst). In contrast, the binding of the Hoechst section is weakened relative to what would have been expected for a free Hoechst-like molecule.

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