Pronounced ^{1}H and ^{31}P NMR Spectral Changes on *meso*-Tetrakis(*N*-methylpyridinium-4-yl)porphyrin Binding to Poly[d(G-C)]·Poly[d(G-C)] and to Three Tetradecaoligodeoxyribonucleotides: Evidence for Symmetric, Selective Binding to 5′CG3′ Sequences

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Abstract: The binding of *meso*-tetrakis(*N*-methylpyridinium-4-yl)porphyrin [TMpyP(4)] to poly[d(G-C)$_2$] and to five synthetic self-complementary oligodeoxyribonucleotides [5′→3′; d(TATATGCGCATATA)$_2$ (I), d(ATATACGCGTATAT)$_2$ (II), d(TATATCGCGATATA)$_2$ (III), d(TATGGGTACCCATA)$_2$ (IV), d(TATATGCATATA)$_2$ (V)] was studied. For poly-[d(G-C)$_2$], I, II, and III, TMpyP(4) binding was accompanied by a characteristic ^{31}P NMR signal at ~-0.9 ppm relative to trimethyl phosphate. Pronounced upfield shifts of some of the imino ^{1}H signals were also observed. In contrast, IV and V did not exhibit these characteristics on TMpyP(4) addition. A related porphyrin species, *meso*-tetrakis(*N,N,N*-trimethylanilinium-4-yl)porphyrin (TMAP), is an outside binder and does not induce these pronounced changes in the spectra of I. Detailed analysis of the imino ^{1}H signals in I–TMpyP(4) and assignment by ^{17}O labeling of the downfield ^{31}P signal to 5′CpG3′ in I–TMpyP(4) support selective symmetric binding at the 5′CpG3′ sequence in I. Less detailed study of II–TMpyP(4) and III–TMpyP(4) and the absence of the effects with IV and V support 5′CpG3′ binding to II, III, and poly[d(G-C)$_2$]. An analogue of I with the C in the 5′CpG3′ binding site replaced by a T, namely d(TATATGTGCATATA)$_2$ (VI), forms a duplex with adjacent GT mismatches. TMpyP(4) does not induce pronounced spectral changes for VI. An unusual feature of the 5′CG3′ TMpyP(4) binding for I, II, and III is that imino ^{1}H signals for two base pairs on either side of the binding site are also shifted upfield. These upfield shifts and the downfield ^{31}P shift are consistent with selective intercalation at the 5′CG3′ sequence by the highly anisotropic porphyrin ring. However, other outside binding sites compete with this most favored binding. Nevertheless, TMpyP(4) binding is unusually selective compared to that of other synthetic molecules that form noncovalent DNA adducts.

Large aromatic porphyrin cations such as *meso*-tetrakis(*N*-methylpyridinium-4-yl)porphyrin [TMpyP(4)] bind to DNA in a complex fashion.[1-12] Many physical techniques, which do not directly identify binding sites, have been used to compare this binding to DNA with that to poly[d(G-C)$_2$] and to poly[d(A-T)$_2$].[8,11,12] These comparative studies have suggested an outside nonintercalative binding to "AT"-rich regions and an intercalative binding to "GC"-rich regions. The specific intercalation site(s) (5′G↓C3′, 5′C↓G3′, 5′G↓G3′) is(are) unknown. Models suggest that TMpyP(4) intercalation is possible at any sequence but only after duplex disruption during insertion.[7,10] Unusually large ^{1}H and ^{31}P spectral shifts presented here identify 5′C↓G3′ as a unique binding site.

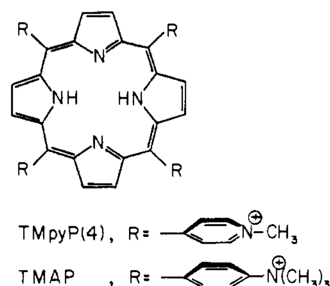
We compared ^{31}P NMR and ^{1}H NMR data for TMpyP(4) binding at several *R* values [ratio of TMpyP(4) to DNA base] to ~200 base pair poly[d(G-C)$_2$][13] and to five synthetic self-complementary (5′→3′) oligodeoxyribonucleotides:[14] d(TATATGCGCATATA)$_2$ (I), d(ATATACGCGTATAT)$_2$ (II), d(TATATCGCGATATA)$_2$ (III), d(TATGGGTACCCATA)$_2$ (IV), and d(TATATGCATATA)$_2$ (V). In addition, we also examined the effect on the ^{31}P and ^{1}H spectra of I of a large porphyrin cation, *meso*-tetrakis(*N,N,N*-trimethylanilinium-4-yl)porphyrin (TMAP), which is believed to be an outside nonintercalative DNA binder[6] (Chart I).

Finally, we studied the effect of TMpyP(4) on the ^{31}P and ^{1}H NMR spectra of the duplex d(TATATGTGCATATA)$_2$ (VI), which is analogous to I but has a T replacing the C at the 5′C↓G3′ binding site.

Experimental Section

Instrumentation. ^{1}H NMR spectra were obtained on a Nicolet 360NB FT NMR spectrometer (NMC-1280 software) with either a standard

Chart I



pulse sequence or a modified 21412 pulse sequence.[15] In the latter case, to minimize the dynamic range problem, typically five sets of 2000 fid

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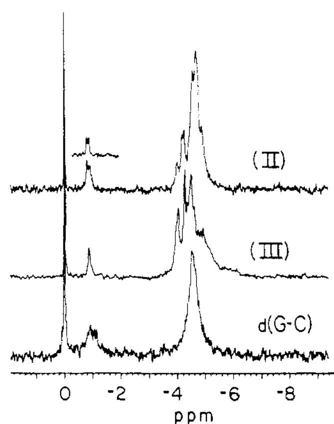

Figure 1. Influence of TMpyP(4) on the 81-MHz ^{31}P NMR spectra of poly[d(G-C)$_2$] (30 °C, R = 0.16) and d(TATATCGCGATATA)$_2$ (III) and d(ATATACGCGTATAT)$_2$ (II) [5 °C, R = 0.06] (R = ratio of porphyrin added per base). Inset above II is for R = 0.03. See the Experimental Section for further details.

(20-bit words) were collected per sample. Each set was block averaged in double-precision mode (40-bit words) and Fourier transformed prior to converting the averaged data back to 20-bit words. The data were collected with 8K data points, a 7042-Hz spectral window, 1-Hz line broadening, and a carrier frequency about 15.4 ppm unless otherwise indicated.

Saturation transfer (ST) and NOE pulse techniques were used in conjunction with the modified ^1H-Redfield 21412 pulse sequence. ST data were collected at 23 °C by using a long (0.5 s) weak monochromatic preirradiation pulse applied at an off-and-on resonance frequency followed by a short delay (5 ms) prior to acquisition. NOE measurements were conducted at low temperature (5 °C) with a stronger preirradiation pulse of 0.1 s and no delay. One hundred transients at each preirradiation frequency were alternately collected and stored. A spectrum (4000 transients, 2-Hz line broadening) obtained with preirradiation on-resonance was subtracted from one with preirradiation off-resonance to obtain ST or NOE difference spectra.

Materials and Methods. PIPES 10 buffer [0.01 M PIPES (Sigma), 0.10 M $NaNO_3$, 0.01 mM EDTA, doubly deionized water] was adjusted to pH 7.00 with NaOH and passed through a 0.22 μm Millipore filter.

Poly(dG-dC)·poly(dG-dC) [poly[d(G-C)$_2$]] from Pharmcia P-L Biochemicals was dissolved in PIPES 10 buffer and sonicated to ca. 200 base pairs (bp) with a microsonication horn as previously described for DNA.[13]

Deoxyoligonucleotides, prepared as previously described,[14] were precipitated twice from ethanol, dissolved in and dialyzed against 1:9 PIPES 10 buffer/H_2O. After ^1H NMR spectroscopy was used to evaluate the sample purity, it was lyophilized and dissolved in H_2O to obtain an undiluted PIPES 10 solution. Concentrations were determined with an ϵ_{260} = 6300 M^{-1} cm^{-1}.

TMpyP(4) tosylate and TMAP chloride were obtained from Mid-Century. TMpyP(4) was converted from the tosylate salt to the chloride salt with an anion-exchange resin (Biorad AG-1 × 8). Porphyrin concentrations were determined spectrophotometrically (ϵ_{424nm} = 2.2 × 10^5 M^{-1} cm^{-1} for TMpyP(4)[10] and ϵ_{412nm} = 4.16 × 10^5 M^{-1} cm^{-1} for TMAP[16]).

The ^1H NMR deoxyoligonucleotide samples had a final concentration of 20 mM in bases except where otherwise indicated and contained PIPES 10, 10% D_2O, and TSP. In most cases the ^{31}P NMR spectra were obtained by inserting the ^1H NMR sample (5-mm tube) into a 10-mm tube containing PIPES 10 buffer and TMP (trimethyl phosphate) as an external reference.

Results

^{31}P NMR. The effects of TMpyP(4) on the ^{31}P NMR spectra of poly[d(G-C)$_2$] and the six deoxyoligonucleotides examined in this study are shown in Figures 1–3. In all cases, in the *absence* of TMpyP(4), the signal(s) is centered at ~−4.0 to −4.2 ppm, relative to TMP. For I–VI, several signals can be resolved but no attempt has been made to assign the signals to a particular phosphate group.

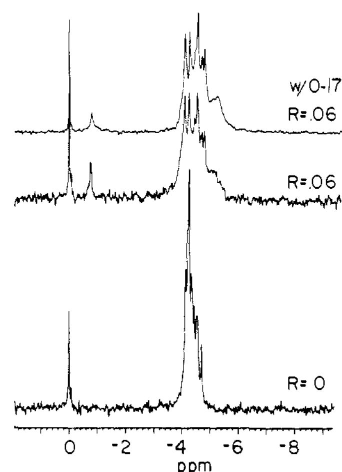

Figure 2. ^{31}P NMR spectra (5 °C) of d(TATATGCGCATATA)$_2$ (I) before (bottom) and after (middle and top) the addition of TMpyP(4) (R = 0.06). In the top trace, the 5′CpG3′ was labeled with ^{17}O, and the spectrum was plotted on the same y scale as used for the middle spectrum.

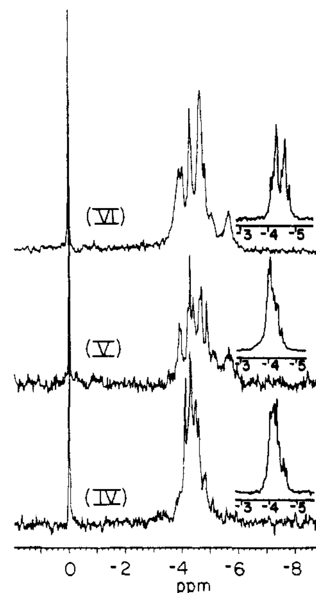

Figure 3. Influence of TMpyP(4) on the ^{31}P NMR spectra (insets: R = 0; reduced y scale) of d(TATGGTACCCATA)$_2$ (IV) [30 °C, R = 0.05] and d(TATATGCATATA)$_2$ (V) and d(TATATGTGCATATA)$_2$ (VI) [5 °C, R = 0.06].

In some cases, in the presence of TMpyP(4), at least one very downfield signal is observed (Figures 1 and 2). In the poly[d(G-C)$_2$] spectrum (Figure 1), the −0.9 ppm signal is the most downfield ^{31}P signal reported thus far for a putative intercalator–DNA complex.[15] The signal, shifted >3 ppm from the normal ~−4.2 ppm region, identifies the binding mode and indicates that TMpyP(4) is in slow exchange among binding sites.[13] Clearly, TMpyP(4) binding to I, II, and III is similar to its binding to poly[d(G-C)$_2$].

The common feature of poly[d(G-C)$_2$], I, II, and III is that there are present 5′C↓G3′ and 5′G↓C3′ binding sites. However, several ^{13}P NMR experiments indicate that the binding site is 5′C↓G3′. First, V contains a 5′G↓C3′ binding site, but there is no characteristic downfield ^{31}P signal. Second, II contains a central 5′G↓C3′ binding site that should give rise to only one ^{31}P downfield signal whereas it also contains two identical 5′C↓G3′ binding sites that could give rise to two ^{31}P downfield signals. Two signals are observed, which are more readily resolved at low R values (Figure 1). Third, IV lacks a 5′C↓G3′ binding site, and no downfield signal was observed on addition of TMpyP(4). Fourth, VI is duplexed

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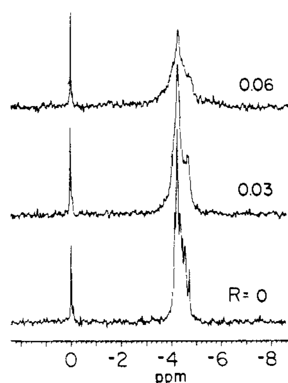


Figure 4. Effect of TMAP on the ^{31}P NMR spectrum of d(TATATGCGCATATA)$_2$ (I) [5 °C].

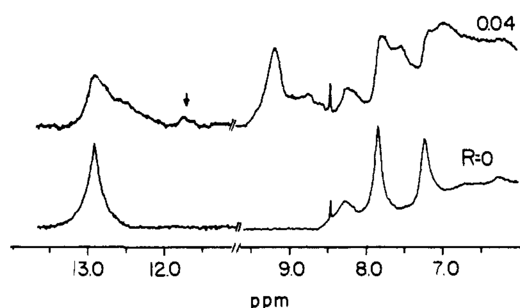


Figure 5. Influence of TMpyP(4) on the low-field region of the 360-MHz ^1H NMR spectrum [30 °C, 90% H_2O Redfield method] of poly[d(G-C)$_2$]. The spectra downfield to 11 ppm are expanded ×3. The arrow indicates the upfield shifted NH signal. The sharp spike at 8.5 ppm is an artifact.

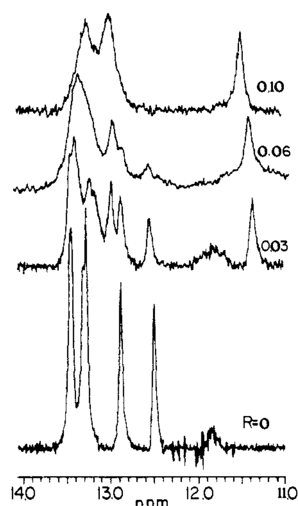


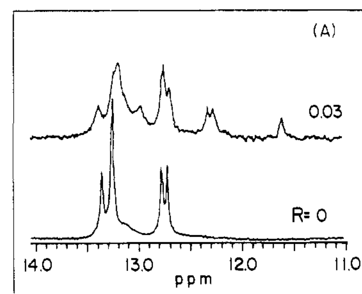
Figure 6. Dependence of the imino ^1H signals of d(TATATGCGCATATA)$_2$ (I) on added TMpyP(4) [5 °C].

(vide infra), is similar in sequence to I, but does not have the 5′C↓G3′ binding site. No downfield signal was observed. Last, the central 5′CpG3′ phosphate group in I was labeled with ^{17}O.[14,17] The diminished area of the downfield peak relative to the main branch of signals at ~−4.2 ppm (Figure 2) establishes that this downfield signal arises from the 5′CpG3′ site.

In contrast to the effect of TMpyP(4), TMAP does not induce a downfield ^{31}P NMR signal on addition to I (Figure 4). This result is consistent with evidence on polymers[8] that the binding modes of TMpyP(4) and TMAP are different.

^{1}H NMR. The major observable effect of TmpyP(4) binding on the NH (imino) ^1H NMR signals of poly[d(G-C)$_2$] (Figure 5), I (Figure 6), and II and III (Figure 7) is to induce new upfield signals at ~11.7–11.4 ppm. All four DNA's have potential 5′C↓G3′ and 5′G↓C3′ binding sites.

Relevant NH signals of I were unambiguously assigned by "melting" and NOE experiments.[18] Competitive outside binding sites on I precluded exclusive formation of the major product I–TMpyP(4). However, ST (Figure 8) and NOE experiments[19] at 23 and 5 °C, respectively, allow assignment of the upfield signal for I–TMpyP(4) at 11.4 ppm to $\check{G}CG\check{C}$ and that at 13.0 ppm to $\check{A}GCGC\check{T}$ (at 5 °C). We are unable to find the signal for the central CG(7) below 9.5 ppm. The simplicity of the I–TMpyP(4) spectrum clearly requires C_2 symmetry (on the NMR time scale) with binding at 5′C↓G3′ (vide infra). Therefore, the NH signals of two bp [GC(6) and AT(5)] to either side of the 5′C↓G3′ binding site are shifted upfield significantly from the original 12.54 and 13.5 ppm shifts in I at 5 °C. Such pronounced changes in the imino signals of next-neighbor bp are unknown for classical intercalators[20,21] and could account for the upfield shoulder for



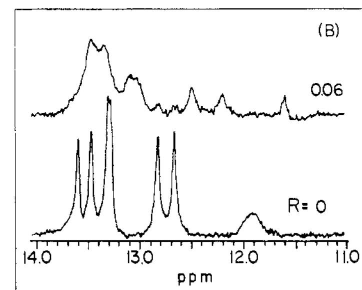


Figure 7. Effect of TMpyP(4) on the imino ^1H signals: (A) d(ATATACGCGTATAT)$_2$ (II) [30 °C]; (B) d(TATATCGCGATATA)$_2$ (III) [5 °C].

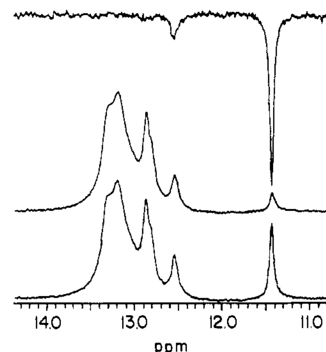


Figure 8. ^1H NMR ST experiment on I–TMpyP(4) [I = d(TATATGCGCATATA)$_2$, 23 °C, 0.07 in nucleobases, R = 0.06]: bottom, preirradiation off-resonance at 12.0 ppm; middle, on-resonance at 11.4 ppm; top, difference spectrum. The 11.4 ppm signal of I–TMpyP(4) correlates with the 12.54 ppm signal assigned to GC(6) of I at 5 °C by NOE methods. Similarly, one overlapping peak at 12.9 ppm at 23 °C was correlated with the 13.0 ppm signal of I–TMpyP(4) at 5 °C (Figure 6). This signal was identified as AT(5) by ST and NOE methods.

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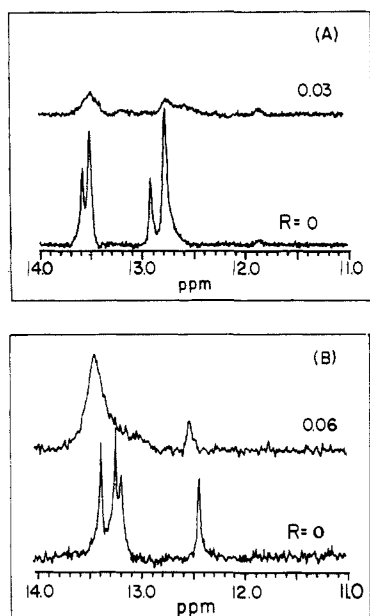

Figure 9. Effect of TMpyP(4) on the imino ^{1}H signals: (A) d(TATGGGTACCCATA)$_2$ (IV), signal area referenced against the *t*-Bu signal of 0.01% *t*-BuOH; (B) d(TATATGCATATA)$_2$ (V).

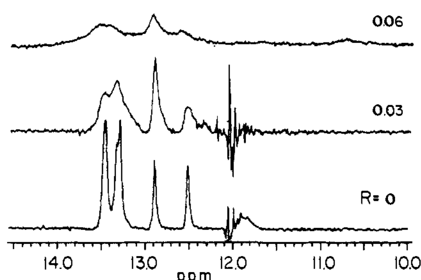

Figure 10. Influence of TMAP on the imino ^{1}H signals of d(TATATGCGCATATA)$_2$ (I) [5 °C]. Carrier frequency is 12.0 ppm.

poly[d(G-C)$_2$]-TMpyP(4) (Figure 5).

The imino ^{1}H signals of II-TMpyP(4) and III-TMpyP(4) (Figure 7) indicate AC↓GCG and TC↓GCG binding sites, respectively. By analogy to I-TMpyP(4), we expect three imino resonances ǍC↓GČǦ and ŤC↓GČǦ upfield to the unresolved AT signals, as observed. Binding at 5′C↓G3′ is further supported by the absence of unusual shifts in the ^{1}H NMR spectra of IV and V, which lack 5′CG3′ (Figure 9). However, there is clear evidence of interaction of TMpyP(4) with IV and V. The principal effect is to diminish the area of the NH signals—an observation consistent either with diminished duplex stability or with an interaction that facilitates H exchange.

Similarly, addition of TMAP to I leads to decreased NH signal intensity. In particular, the NH signal assigned to GC(6) is preferentially decreased relative to that assigned to GC(7) on the addition of TMAP (Figure 10). Although we did not investigate this outside binder in detail, it is obvious that it can have a selective effect.

Discussion

We asserted above that the NH signals attributed to I-TMpyP(4) indicate C_2 symmetry on the NMR time scale. For self-complementary duplexes (C_2 symmetry), two equivalent bp give rise to each NH signal. Any interaction that breaks this symmetry would lead to a species with potentially twice the number of NH signals. Evidence for such complexity can be found in the spectra of II-TMpyP(4) and III-TMpyP(4) (Figure 7) where the noncentral location of the 5′C↓G3′-found TMpyP(4) breaks C_2 symmetry. Furthermore, the highly anisotropic TMpyP(4), if symmetrically intercalated at 5′TGC↓GCA3′ of I, should cause a large downfield shift of the 5′CpG3′ ^{31}P signal. The signal at -0.8 ppm is, in fact, assigned to 5′CpG3′ by ^{17}O labeling[14,17] (Figure 2). Since the phosphate groups on both chains are identical (i.e., the seventh phosphate group from either end), one ^{31}P NMR signal is expected. However, the two phosphate groups at the 5′C↓G3′ binding site will be inequivalent in II-TMpyP(4) and III-TMpyP(4) (i.e., for II, one group is sixth from the 3′ end of the strand in an ACGC sequence and the other the eighth from the 3′ end in a GCGT sequence). Two ^{31}P signals might be observed. This result is found for II-TMpyP(4), confirming our interpretation (Figure 1). However, two signals are not resolved at ca. -0.8 ppm for III-TMpyP(4). The similarity of the ^{31}P shifts at ca. -0.8 ppm for all three TMpyP(4) adducts of I, II, and III suggests that this shift is insensitive to sequence. Therefore, the observation of a single downfield ^{31}P signal, unless accompanied by signal assignment (e.g., ^{17}O labeling), cannot be taken as evidence for symmetric binding.

If it is assumed that the NH groups lie above the center of the porphyrin ring in I-TMpyP(4), the GN(1)H of GC(7) and GC(6) would be at 3.4 and 6.8 Å and the TN(3)H of AT(5) at 10.2 Å, respectively, above the center. Upfield shifts of ~4, ~1.3, and ~0.4 ppm, respectively, would be expected from the shift map for tetraphenylporphyrin.[22] The GC(6) and AT(5) shifts of 1.2 and 0.5 ppm, respectively, are close to those expected from this preliminary model. The GC(7) shift is predicted to be in the region of ~9 ppm where the strong TMpyP(4) and nucleobase CH resonances are found. The GC(7) signal would overlap with the large broad TMpyP(4) signals, possibly accounting for our failure to observe it directly.

CD studies of the Soret band indicate "AT" binding leads to positive and "GC" binding to primarily negative induced peaks.[8,10] Calf thymus DNA, with 42% GC content, induces both CD characteristics even at low $R < 0.03$.[7,10] Such spectral data and numerous other studies have led to the hypothesis of competitive binding between "GC" intercalation and "AT" outside binding.[10,11]

Likewise, numerous observations we have made indicate that secondary sites on I are occupied by TMpyP(4). For example, the induced CD spectrum of I-TMpyP(4) ($R = 0.06$) has both negative and positive peaks. At $R = 0.03$, only 50% of the unique 5′C↓G3′ binding is observed by ^{1}H NMR, although enough TMpyP(4) is present to occupy 84% of this site. At $R = 0.10$, all the GC(6) imino signal at 12.54 ppm (at 5 °C) is finally eliminated (Figure 6) but new ^{31}P signals upfield to -5.0 ppm and severely broadened CH signals are evident. Addition of $Mg(NO_3)_2$ to 0.05 M does not significantly restore the 12.54 ppm signal. This finding and the expected high binding constant ($>10^4$)[12] exclude incomplete TMpyP(4) binding. The broadening and new signals preclude interpretation of the CH signals and meaningful integration of the ^{31}P signals of solutions of I and TMpyP(4).

Although our failure to observe the GC(7) signal can be attributed to its overlap with the TMpyP(4) signals, we cannot exclude a symmetric 5′C↓G3′ binding model with disruption of hydrogen bonding between the GC(7) bp. In this case, the NH signal could be lost due to solvent exchange. The binding of TMpyP(4) to VI was studied as one test of this model. Evidence exists that TMpyP(4) prefers to stack with two G residues from dGMP in preference to two C residues from dCMP.[23] Stacking to two T residues of dTMP is favored somewhat over two C residues of dCMP. The driving force for the specific 5′C↓G3′ interaction could be stacking to the two G's on opposite strands. In such a case, TMpyP(4) could bind strongly to VI. However, no specific interaction was observed (Figure 11).

Our failure to observe similar binding of TMpyP(4) to I and VI does not exclude disruption of H bonding between near-neighbor bp. Indeed, if such disruption extended to the next-

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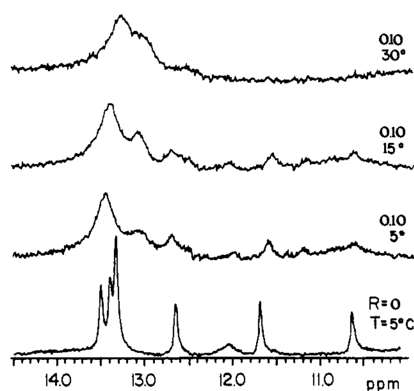

Figure 11. Effect of TMpyP(4) on the imino ^{1}H signals of d(TATATGTGCATATA)$_2$ (VI). The two imino signals of GT(7) are the two sharp signals to highest field. The similarity of the signals for AT(2)→GC(6) to that of d(TATATGCGCATATA)$_2$ (I) at R = 0 demonstrates that I and VI form similar duplexes, except for the central bp (7).

neighbor, bp, the upfield shift of the GC(6) NH signal might be explained since non-H-bonded G species have N(1)H signals at ~11 ppm.[24] However, we feel this explanation of the GC(6) NH shift in I–TMpyP(4) has several problems. First, GN(1)H exchanges very readily with H_2O.[24] However, with increasing temperature, the GC(6) NH signal of I–TMpyP(4) diminishes in intensity somewhat less than the GC(6) NH signal in I. Second, the TN(3)H signal of ǍC↓G in II–TMpyP(4) or ŤC↓G in III–TMpyP(4) is expected to be at ca. the same shift as the GN(1)H signal of C↓GČ in these adducts if base pairing is disrupted, since these signals for monomers are readily observed and have roughly the same chemical shift.[24,25] Third, the most plausible reason for not observing GC(6) NH exchange with H_2O for I–TMpyP(4) by this alternative model would be that the NH group is protected in a duplex. In such a case, we would have expected base stacking to shift the NH signals to higher field than 11 ppm[26] —not downfield at ~11.5 ppm as observed. Thus, at this juncture, our data suggest a binding mode in which the next neighbor bp are still hydrogen bonded. Furthermore, the observation of the GC(6) NH signal in I–TMpyP(4) and the ST experiment depicted in Figure 8 require that the interconversion of I and I–TMpyP(4) proceeds without appreciable NH–H_2O exchange.

The specific details of the interaction of the TMpyP(4) with the near-neighbor bp and the evaluation of the retention of H bonding by these base pairs must await the discovery of an even more selective interaction. However, for a species that adds to DNA noncovalently, the synthetic TMpyP(4) cation exhibits unprecedented selectivity for 5′CG3′ over 5′GC3′, 5′GG3′, 5′TG3′, 5′GT3′, and 5′GA3′ sequences. The complex natural product, actinomycin D, is the only intercalator known to exhibit pronounced specificity for one site (5′GC3′).[17a,21,27] However, this anticancer antibiotic binds to other dinucleotide sequences containing G. Although this study provides the first direct evidence consistent with intercalation of TMpyP(4) at "GC" but not at "AT" sites, more studies are needed to further define the adducts formed by DNA binding porphyrins.

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Registry No. 2I·TMpyP(4), 102573-50-2; 2II·TMpyP(4), 102573-51-3; 2III·TMpyP(4), 102573-52-4; IV, 102505-17-9; V, 102520-98-9; VI, 102505-18-0; TMpyP(4), 38673-65-3; TMAP, 69458-19-1; TMpyP-(4)·2poly[d(G-C], 102535-16-0.

(24) In unpublished studies, we find that cGMP(2′,3′) has an N(1)H signal at 11.0 ppm in H_2O at pH 6.4–4.0. Under similar conditions, the spectrum of TMP has an N(3)H signal at 11.1 ppm for pH 4.9–2.5.

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Prostacyclin: Evidence That Intramolecular General-Acid Catalysis by Its Carboxylic Acid Group Is Responsible for the Extra Hydrolytic Lability

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Abstract: Rates of hydrolysis of the vinyl ether functional group of prostacyclin and its methyl ester were measured in aqueous solution at 25 °C over the acidity range $-\log[H^+]$ = 1–8. The rate profile for prostacyclin shows a break, from which pK_a = 5.03 ± 0.15 may be inferred for the carboxylic acid group of this molecule and a 10^2-fold greater reactivity may be deduced for the carboxylate over the carboxylic acid form. The hydrogen ion catalytic coefficient for reaction of the carboxylic acid form, k_{H^+} = 439 ± 4 M^{-1} s^{-1}, is similar to that of prostacyclin methyl ester, k_{H^+} = 418 ± 5 M^{-1} s^{-1}, and is normal for a vinyl ether of this structure. Kinetic isotope effects and unusually weak catalysis by external general acids suggest that the abnormal reactivity of the carboxylate form is the result of intramolecular general-acid catalysis by the carboxylic acid group; an effective molarity of 0.6 M can be estimated for this process.

Prostacyclin (**1**, R = H; Scheme I) is a recently discovered[1] eicosanoid with remarkable anti-blood-clotting properties.[2] This gives it great potential as a therapeutic agent for the treatment of thrombosis and also as an anticlotting factor to confer non-

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