Covalent binding of a bridged pyridinium aldehyde with the self-complementary decamer [d(ATGACGTCAT)]$_2$. Gel analysis, MALDI mass spectrometry and NMR studies

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Gel analysis, MALDI-TOF mass spectrometry and NMR spectroscopy show that a tethered pyridinium aldehyde, from a new class of DNA-interacting agents, binds to the self-complementary [d(ATGACGTCAT)]$_2$ decamer. The reaction proceeds *via* a two-step pathway: fast non-covalent outside association (complex formation) is followed by slow covalent addition (adduct formation) upon temperature increase. Both interactions occur mainly at the central CpG in the oligonucleotide, the latter leading finally to the reversible formation of an aminal through nucleophilic attack of the exocyclic guanine amino group in the minor groove of the helix.

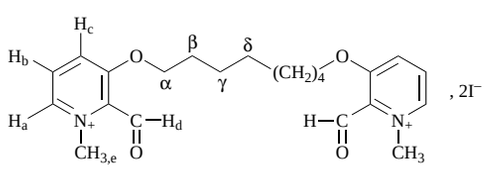
Bridged pyridinium aldehydes (BPA), a newly designed series of bifunctional derivatives, have been shown to bind DNA *in vitro* and to induce growth inhibition in cultured tumor cells (L1210 and C38 lines).[1,2] Binding involves the carbonyl groups in BPA ligands and is reversible, though presumably covalent. As a consequence of release of DNA topological constraint, it induces a sharp decrease of supercoiled plasmid pBr322 DNA electrophoretic mobility. Inhibition of DNA cutting by restriction endonucleases whose recognition sequences contain CG pairs (whereas normal digestion by appropriate enzymes occurs when these nucleotides are absent from the intervening sequence) strongly suggests a base- or sequence-specific reaction. It was also observed that the tether linking the two pyridinium moieties facilitates the reaction, possibly by opening the DNA helix, thus exposing the nucleic bases to the ligand.[1] The potential interest of their biological properties, which are probably mediated by their interaction with DNA, prompted us to further examine the reaction of BPA derivatives at the molecular level.

The self-complementary decamer [d(ATGACGTCAT)]$_2$ was chosen in this study as a model for DNA for the following reasons. (i) The central 5′-CpG-3′ step has been recognized to present significant distortion with respect to a canonical B-DNA structure,[3] (ii) 5′-ApC-3′ and 5′-CpA-3′ sequences may induce transient kinks.[4,5] These factors point towards possibly significant local flexibility of the double helix which would be favorable to reactions involving destabilizing base pairs. It is also worth noting that the central octanucleotide in the decamer is the cyclic AMP response element, the target of activation factors in the series of leucine zipper proteins,[3,6] and that CpG motifs flanked by two 5′ purines and two 3′ pyrimidines are expected to activate immune defenses.[7] Hence, this investigation may provide information relevant to the understanding of the physiological effects of BPA derivatives.

In this study polyacrylamide gel electrophoresis is used to visualize the altered mobility of BPA-treated oligonucleotide with respect to that of the free decamer. The formation of stable entities together with the estimate of their mass is assessed by means of MALDI-TOF mass spectrometry. NMR spectroscopy provides information about functional groups involved in the interaction and base specificity. In the BPA series, only the member with an eight methylene linking tether (referred to as BPAC$_8$) is considered in this study.[1]

Experimental

BPAC$_8$ and the self-complementary sequence, d(ATGACGTCAT), are numbered as shown in Scheme 1. The oligonucleotide was provided as the ammonium salt (18 ammonium ions per duplex; molecular mass 6356 Da) by the Institut Pasteur (Paris). BPAC$_8$ was prepared and purified by published procedures.[8] The reactions of the ligand with the oligomer were run at both 25 and 38 °C, at which temperatures the decamer retains its double-stranded structure (T_m = 55 °C).[9]



Duplex decamer sequence numbering used in molecular model

5′ - A1	T2	G3	A4	C5	G6	T7	C8	A9	T10 - 3′
3′ - T10′	A9′	C8′	T7′	G6′	C5′	A4′	G3′	T2′	A1′ - 5′

Scheme 1

Gel electrophoresis

In order to render the BPA–oligonucleotide adducts amenable

to gel analysis, the decamer (referred to as CRE) was first oligomerized. CRE was treated with polynucleotide kinase (Pharmacia) in an appropriate incubation buffer for 30 min at 38 °C. T4 DNA-ligase (Boehringer–Mannheim) was introduced into the reaction vessel and was allowed to react for 1 h at 38 °C. The reaction mixture was then treated with an equal volume of phenol–chloroform and precipitated by ethanol in the presence of 0.3 M sodium acetate. A ladder of CRE oligomers (polyCRE) was observed on native 13% polyacrylamide gel [running buffer: 45 mM tris-borate, 1 mM ethylenediaminetetraacetic acid (EDTA), pH = 8] and was compared with a ladder of the polymerized 5′-GAAAACGTTTTC-3′ dodecanucleotide (prepared as above) in order to assess the number of monomer units in the various polymers (the migration distances coincide when the degree of oligomerization of the dodecamer is 5 and that of the decamer 6). Typically, a 2.5 μg μl^{-1} (corresponding to a nucleotide concentration of 7.6 μmol μl^{-1}) solution of polyCRE in 10 mM cacodylate buffer, pH = 7.4, was reacted with $BPAC_8$ at 38 °C for times ranging from 0 to 20 h and at ligand/nucleotide molecular ratios, *r*, varying from 0 to 5. After incubation, the mixture was precipitated by ethanol, redissolved in Tris-EDTA buffer at 4 °C, and precipitated again. No $BPAC_8$ could be detected by UV in the supernatant after this second precipitation. After electrophoresis, the gels were digitized photographically and the lanes scanned. The migration distances of free polyCRE were first fitted to the mass of the various oligomers by means of a non-linear regression, starting from a mass of 6032 Da (corresponding to the mass of the anionic moieties of the ammonium salt) when the degree of oligomerization, *n*, is 1 and adding a mass of 6032 + 77 Da (77 Da arising from addition of a phosphate group and elimination of one water molecule which have occurred in the polymerization process), when *n* is incremented by 1. This non-linear fitting (see Supplementary Material,† Table S1) was subsequently used to determine the mass of the various $BPAC_8$-treated oligomers.

MALDI-TOF mass spectrometry

Spectra were recorded using a home-built time-of-flight mass spectrometer operated in the negative mode. A mixture of 90% 2,4,6-trihydroxyacetophenone and 10% ammonium citrate was used as matrix. The analyte to matrix molar ratio was approximately 1/10 000. A 2 μl aliquot of the NMR samples in H_2O–D_2O was used.

NMR spectroscopy

1D and 2D proton NMR spectra were recorded with a Bruker AM-500 MHz spectrometer (D_2O experiments) and a Bruker DMX-500 MHz spectrometer (H_2O–D_2O 90/10 experiments). The temperatures were 298 and 283 K (non-exchangeable and amino–imino proton investigations, respectively). In the H_2O–D_2O experiments, the water resonance was suppressed by a 'WaterGate' sequence using field gradients[10] associated with a 3–9–19 pulse sequence for the selective excitation of water (SW = 20 ppm, delay = 175 μs for the free decamer or 80 μs for the adduct). Presaturation of the residual HOD signal in D_2O was achieved using low power irradiation. All two-dimensional homonuclear spectra were acquired in the phase-sensitive mode using the TPPI mode to achieve quadrature detection in the evolution dimension.[11]

Samples were prepared by dissolving the CRE decamer (5.5 mg) in 450 μl of phosphate buffer (pH = 7.4, *I* = 50 mM) containing 1 mM EDTA. In the non-exchangeable proton investigation, the sample was first lyophilized from the aqueous buffer, then twice from 99.9% D_2O, and finally dissolved in 400 μl of 99.95% D_2O.

The CRE decamer was mixed with 1–3 equiv. of $BPAC_8$ (only the results for 3 equiv. will be reported here). Two NMR experiments were run successively at 25 and 38 °C. The mixture was first allowed to stand from 0 to 36 h at 25 °C and its evolution was monitored by 1D NMR in D_2O (spectral width 4.4 kHz over 16 k data points, relaxation delay 2.0 s) and H_2O–D_2O (90/10) (spectral width 10 kHz over 32 k data points, relaxation delay 2.0 s). Additionally, NOESY correlations were recorded in both D_2O (at 298 K) and H_2O–D_2O 90/10 (at 283 K). NOESY experiments were performed on non-spinning samples, with 2048 points in t_2 and 320 points in t_1. In each experiment, 160 scans were collected over a spectral width of 4.4 kHz (D_2O) or 10 kHz. (H_2O–D_2O) with a relaxation delay of 2.0 s and a mixing time of 200 ms. The spectra were zero-filled to 2048 points in t_1 prior to Fourier transformation. The data were then processed with a sine-squared 90° phase-shifted function in both dimensions.

In a second step, the temperature was raised to 38 °C. NOESY spectra were recorded in H_2O–D_2O at various time intervals (30 min, 2 and 20 h). All NMR parameters were as above except for the number of scans which was increased to 384.

In another experiment, the decamer was treated with a large excess of ligand (15 equiv. per duplex) for 15 h at 38 °C. The reaction mixture was then ethanol-precipitated in the presence of 0.3 M sodium acetate, resuspended in cold phosphate buffer and precipitated again. After centrifugation, the pellet was dissolved in fresh H_2O–D_2O, and 1D and NOESY spectra were run immediately at 283 K using the usual procedure. After the sample had been allowed to stand at 38 °C for 24 h the NMR spectra were recorded again. Because of decreased spectral resolution and scarce NOESY crosspeaks, (Table 1), no attempt to quantify through-space interaction distances in the adduct was made.

Table 1 Proton chemical shifts of free $BPAC_8$ (in ppm) and $BPAC_8$–DNA connectivities for the complex and the adduct. Recorded in D_2O at 298 K

$BPAC_8$ proton	δ	complex	adduct[a]
H_a	8.34	H4′C5	H4′C5
			H4′C8a
H_b	7.92	AC8	H2′/H2″G6a
			H1′G3
H_c	8.13	H6C5	H1′C5
		H4′C5	H4′C5
		H4′C8	H4′C8
			AC8
H_d	6.96	H5C8	H4′T2
		H4′C8	H4′C5
			H5C8
			H4′C8
H_e	4.59		H8G6
$CH_2\alpha$	4.28	H8G3	H8A4
		H6C5	H4′C5
		H8G6	H6C8a
		H6C8	AC8
		AC8	
$CH_2\beta$	1.74		
$CH_2\gamma,\delta$	1.39–1.28	H2A9	H8G6a

[a] 'a' for 'adduct'.

Results

NMR analysis

Prior to the NMR studies of the $BPAC_8$–CRE interaction, NMR investigation of the free decamer had been performed (see full details for assignment of resonances, chemical shifts in Tables S2–S3 and Figures S1–S3 in the Supplementary

† Supplementary data (SUPPL. No. 57302, 20 pp.) has been deposited at the British Library. For details of the Supplementary Publication Scheme, see 'Instructions for Authors', *J. Chem. Soc.*, *Perkin Trans. 2*, 1997, Issue 1.

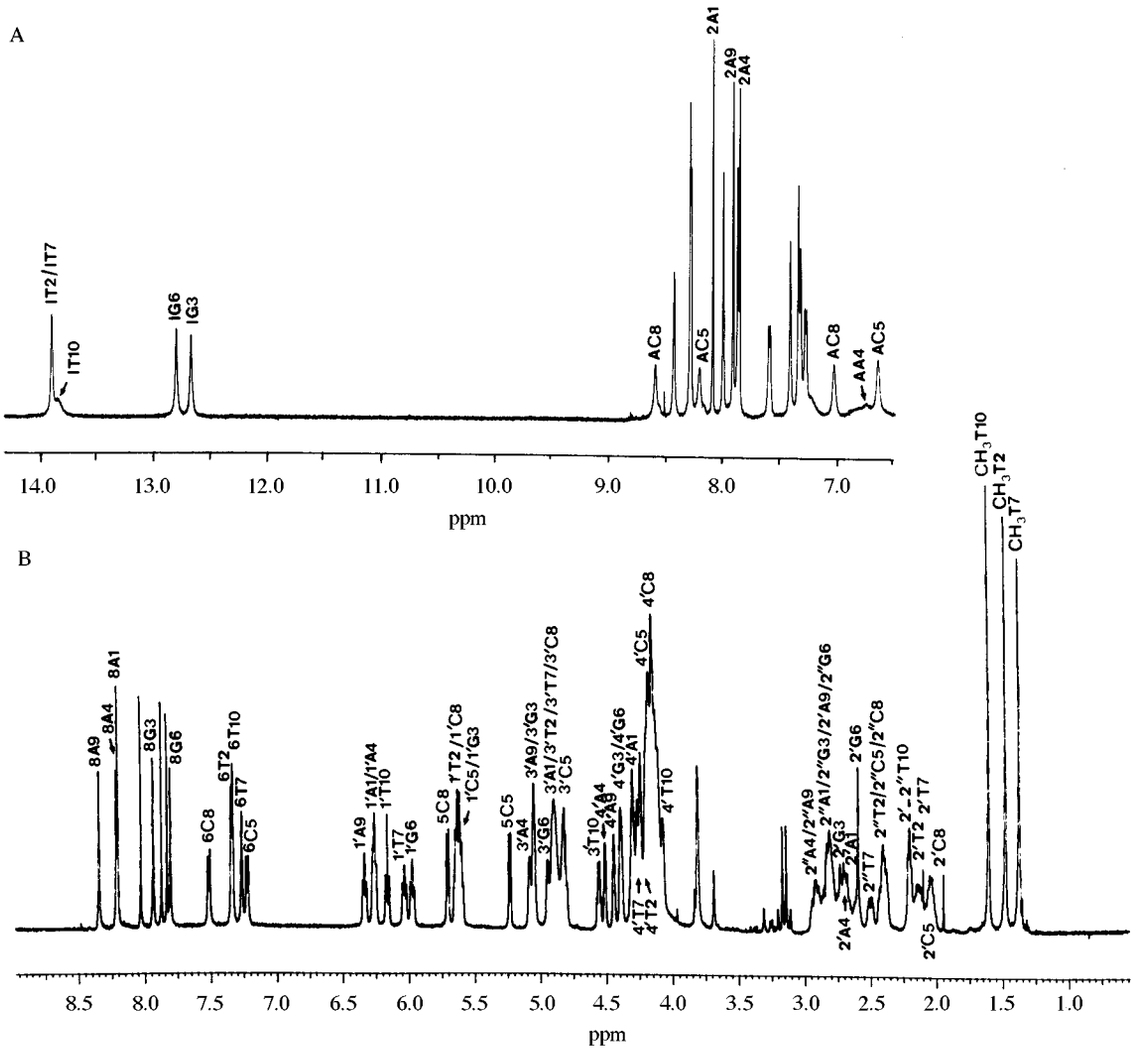


Fig. 1 One-dimensional 500 MHz ^{1}H NMR spectra of the free oligomer: (A) in H_2O–D_2O (90/10) at 283 K; (B) in D_2O at 298 K

Material). Only 1D spectra in D_2O and H_2O–D_2O are shown here (Fig. 1).

Non-covalent interaction at room temperature. The duplex is progressively titrated by increasing amounts of $BPAC_8$ at 25 °C to the final ligand/decamer molar ratio of 3/1. 1D and 2D spectra (recorded at 283 and 298 K) show significant spectral changes, occurring immediately after ligand addition, and indicating rapid formation of a complex. No further evolution in the spectra is observed even after 36 h. Most interestingly, the addition of $BPAC_8$ causes small frequency shifts or line broadening preferentially at certain sites in the oligomer. For example, H6T7 is shifted 0.1 ppm downfield without significant loss of resolution, H8G6 is only slightly shifted and overlapped with the Hb proton of $BPAC_8$ while H6C5 and H6C8 as well as H5C5 and H5C8 experience dramatic loss of resolution (Fig. 2). NOESY correlations recorded in D_2O (298 K) at a $BPAC_8$/decamer ratio of 3/1 show many ligand-CRE connectivities (Table 1). Remarkably, the intranucleotide NOE crosspeaks between H8G6 and G6 sugar protons, which were found to be intense in the spectrum of the free oligomer, are absent from the complex. This cannot arise from a general decrease of signal intensities since, for example, the H5C5–H6C5 crosspeak is present on the 2D spectrum of the complex, despite the very low intensities of these resonances at 298 K.

Besides, no correlation between $BPAC_8$ protons and exchangeable protons of the decamer can be detected from NOESY spectra obtained in H_2O–D_2O (283 K). Moreover, no significant alteration of amino–imino proton resonances (in the 12–15 ppm range) with respect to the free decamer is observed [Fig. 3(A)]. Since the imino protons are known to be very sensitive to fraying and helix distortions, this indicates no marked structural changes in the decamer upon interaction with BPA and, hence, rules out formation of a covalent entity.

Finally, when after 36 h at 25 °C the $BPAC_8$–CRE mixture is ethanol-precipitated, redissolved in cold buffer, precipitated again and then redissolved, its 1D NMR spectrum is identical to that of the free decamer with a fully restored resolution.

Covalent binding interaction at 38 °C. In a second series of experiments, where $BPAC_8$ and CRE (in a ratio of 3/1) were allowed to react at 38 °C, the 1D and 2D NMR spectra (283 K) recorded in H_2O–D_2O (90/10) show marked changes with respect to their counterparts from reaction at room temp. (Fig. 4). The most conspicuous new features are: (i) the appearance of new peaks growing with time [labelled 'a' for 'adduct' in Fig. 3(B) and 4] in the amino region around 10 ppm and in the imino region around 13.5 ppm; (ii) the new amino lines are not affected by presaturation of the water resonance (whereas the intensities of the other exchangeable protons become vanishingly small, thus clearly indicating that these peaks arise from non- or slowly-exchangeable protons); (iii) the appearance of correlations between the 13.5 and 10 ppm resonances in the NOESY spectrum (whereas the homologous region in the NOESY map of the complex formed at room temp. is empty) [Fig. 3(B), boxed area]. Examination of the 1D and 2D spectra [Fig. 3(B) and 4] clearly indicates the formation of several adducts, one of them being largely predominant [referred to as G6a maj., Fig. 4(B)]. The 2D spectrum shows that the large resonance at 13.38 ppm, which is correlated with those of the C5 amino protons at

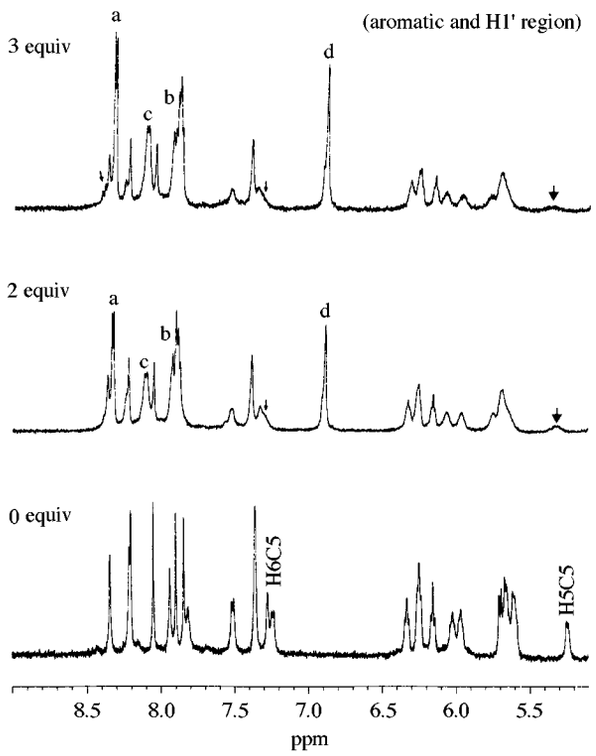


Fig. 2 One-dimensional 500 MHz ^{1}H NMR spectra (298 K) of $BPAC_8$–CRE mixture at room temperature for ligand–decamer ratios of 0 to 3/1. Proton chemical shifts of $BPAC_8$: $H_a = 8.34$, $H_b = 7.92$, $H_c = 8.13$, $H_d = 6.96$, $H_e = 4.59$, $CH_2\alpha = 4.28$, $CH_2\beta = 1.74$, $CH_2\gamma = 1.39$ and $CH_2\delta = 1.28$.

8.18 ppm (hydrogen-bonded amino proton) and 6.63 ppm, can be assigned to the ligand-modified G6 imino proton (IG6a), whereas the much weaker peak at 13.47 ppm, correlated with the C8 amino protons (8.59 ppm for the hydrogen-bonded amino proton and 7.06 ppm), is likely to be that of ligand-modified G3 imino proton (IG3a). These protons are shifted downfield by 0.6 and 0.8 ppm with respect to their positions in the unmodified duplex and, as seen above, are correlated with the ligand-modified amino protons of guanines which are non-exchangeable upon $BPAC_8$ addition. Observation of AG6a and AG3a (weak) resonances, even when the residual HOD signal is suppressed by presaturation is evidence for covalent attachment of $BPAC_8$ with the duplex. This result clearly shows formation of an adduct which stabilizes the duplex.[12a] Assignments in the exchangeable proton region are given in Fig. 3(B) (squared area on the map).

In the region of the NOESY map showing aromatic to H1′, H3′, H4′, H2′/H2″ and methyl connectivities, a few resonances are duplicated. This duplication corresponds to the displacement of the proton resonances due to adduct formation [for example: H6T2, H8G6, H5/H6C8, H8A9 and both the free and hydrogen-bonded protons in NH_2C5 (all labelled 'a')]. The doubling of these resonances is due to the loss of two-fold helical symmetry of the decamer duplex as a result of covalently linking $BPAC_8$ to one strand. This spectral feature was not observed for the non-covalent interaction at room temperature (see above). The 3/1 excess of ligand makes it impossible to identify more than 19 NOE contacts unambiguously, most of them also being present in the NOESY map of the complex (Table 1).

When the decamer is exposed to a 15-fold $BPAC_8$ excess and then twice ethanol-precipitated, the NMR spectra run immediately after redissolution are identical to those at a ligand/decamer ratio of 3/1. However, when NMR is performed after the sample has been heated for 24 h at 38 °C, the resonances at 10 ppm, assigned to the amino protons of the ligand-modified guanines, become vanishingly small.

Attempts to acquire information on adduct formation from ^{31}P NMR failed due to considerable loss of resolution.

Polyacrylamide gel analysis

Significant electrophoretic retardation builds up with time (Fig. 5) and increasing ligand concentration (not shown) in $BPAC_8$-treated oligomers. Quantification of migration distances after a 20 h reaction by means of a non-linear regression (lane 7 in Fig. 5 and Supplementary Material, Table S1) shows that retardation is satisfactorily accounted for by a constant mass increase of 540 ± 175 Da per decameric unit in the oligomer. Since the molecular mass of $BPAC_8$ is 640 Da, with iodide counter-ions included (513 and 386 Da for the mono- and di-cations, respectively), this suggests formation of an adduct with 1 : 1 stoichiometry. Electrophoresis run after sample treatment for 3 min at 95 °C in 0.2 M NaOH shows that the adducts are not stable under these conditions and that polyCRE does not retain its double-stranded structure. This is evidence for the absence of stable cross-linking.

MALDI-TOF mass spectrometry

The MALDI mass spectrum of the free CRE decamer (appearing as a single strand with a major peak at *m/z* of 3026, which corresponds to the mass of the acidic oligomer formed in the matrix upon substitution of the ammonium ions by protons) is first run as a control (Supplementary Material, Fig. S4). After $BPAC_8$ and CRE in a ratio of 3/1 have been allowed to react at 38 °C for 20 h, a new peak centred at *m/z* 3368 appears together with minor ones. This new peak can be resolved into three main components, one of them culminating at *m/z* 3412, the expected mass for the CRE–$BPAC_8$ adduct (3026 + 386 = 3412 Da). No peak corresponding to cross-linked strands in the range of *m/z* 6000 is observed. Ethanol precipitation followed by resuspension in buffer yields to the same mass spectrum whereas subsequent incubation of the sample at 38 °C for 24 h shows the reversible character of the adducts (Supplementary Fig. S4 C).

Discussion

This study has shown that tethered pyridinium aldehydes interact with the decanucleotide CRE in a two-step process leading finally, when temperature is raised to 38 °C, to a covalent adduct. When $BPAC_8$–oligonucleotide mixtures are allowed to interact at room temperature, even for several hours, no stable molecular entities involving both components can be detected either by electrophoresis or by mass spectrometry, only the free decamer being observed. NMR analysis under the same conditions, however, clearly shows that the ligand–CRE interaction does take place and leads to immediate formation of a complex. The lability of this complex is confirmed by the fact that the spectrum run after alcohol precipitation is that of the free oligomer. Numerous kinetic data on non-covalent binding of cationic ligands to DNA support the idea of a rapid equilibrium between $BPAC_8$ and CRE, which results in broadening of resonance lines at the sites of contact. Scarce NOESY cross-peaks preclude any clear-cut image of the non-covalent complex, built up from NOE distances, to be proposed. However, NOE correlations reported in Table 1 indicate that the ligand interacts mainly with aromatic and 4′ sugar protons in the decamer thus suggesting that the complex results from outside binding arising from Coulomb interaction between the phosphate groups and the cationic ligand. Preferential binding at the central CpG step, (shown by specific broadening of the H5C5 and H6C5 resonances in the 1D spectrum and disappearance of the intraresidue crosspeaks involving mainly H8G6 and its sugar protons in the NOESY correlation), and, to a lesser extent, at C8, may be understood as follows. Molecular modelling of the free decamer under NMR constraints (see Sup-

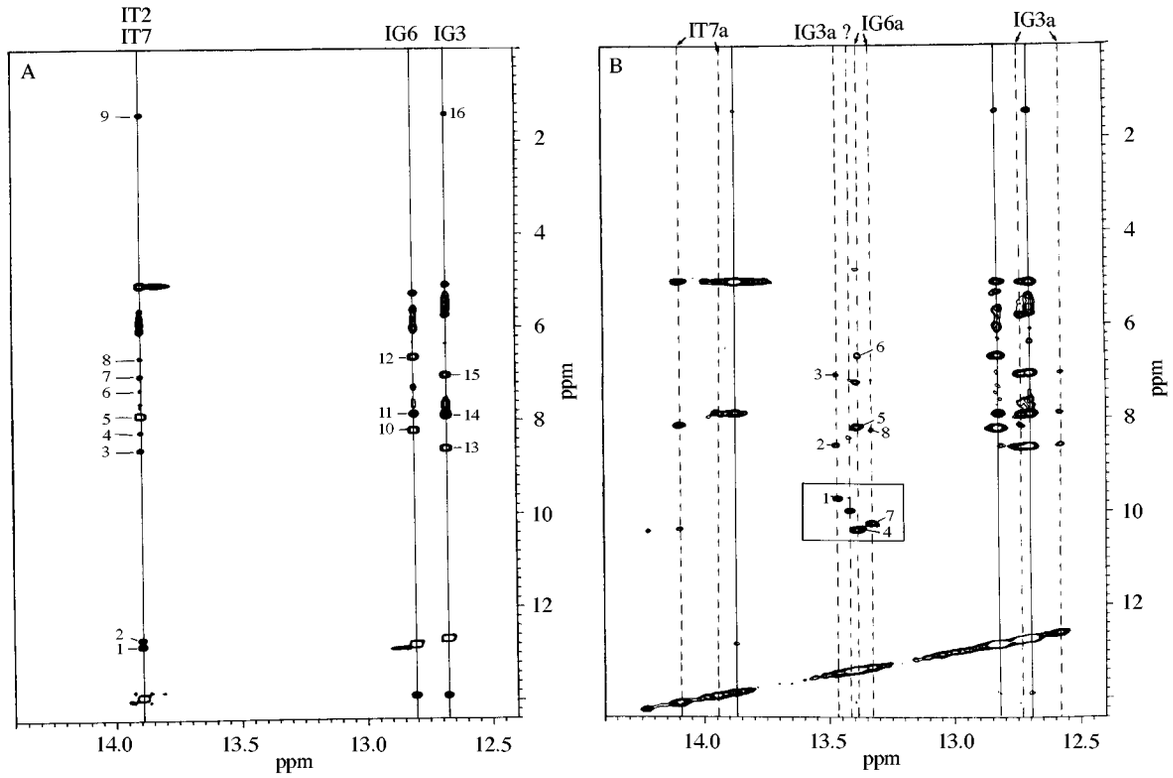


Fig. 3 NOESY spectra (283 K) in H_2O–D_2O (90/10) showing the connectivities of imino protons: (A) $BPAC_8$–CRE (3/1 ratio) reacted at room temperature; (B) $BPAC_8$–CRE (3/1 ratio) reacted at 38 °C for 2 h. Solid vertical lines correspond to the free decamer resonances. Dotted vertical lines correspond to the $BPAC_8$–CRE adduct resonances. Spectrum (A) **1**, IT7-IG6; **2**, IT2-IG3; **3**, IT7-AC8H-bonded; **4**, IT7-AC5H-bonded; **5**, IT2-H2A9(i) and IT7-H2A4(i); **6**, IT2-AA9(i); **7**, IT7-AC8; **8**, IT7-AC5(i); **9**, IT2-CH_3T2 and IT7-CH_3T7; **10**, IG6-AC5H-bonded(i); **11**, IG6-H2A4(i); **12**, IG6-AC5(i); **13**, IG3-AC8H-bonded(i); **14**, IG3-H2A9(i); **15**, IG3-AC8(i); **16**, IG3-CH_3T2. Spectrum (B) boxed area corresponds to imino–amino connectivities of the adduct. **1**, IG3a-AG3a; **2**, IG3a-AC8H-bonded(i); **3**, IG3a-AC8(i); **4**, IG6a *-AG6a, **5**, IG6a *-AC5H-bonded; **6**, IG6a *-AC5; **7**, IG6a-AG6a; **8**, IG6a-AC5H-bonded. (i) interstrand connectivities, * major peak.

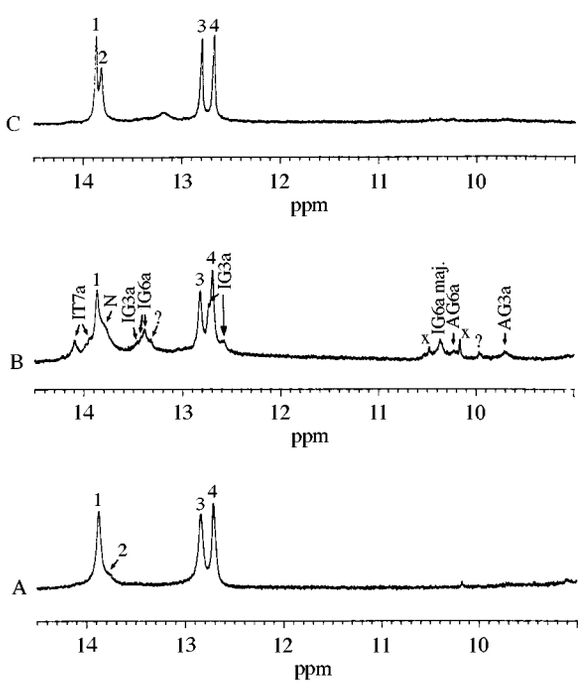


Fig. 4 One-dimensional 500 MHz ^{1}H NMR spectra (283 K) of the $BPAC_8$–CRE (3/1 ratio) in H_2O–D_2O (90/10) in the amino–imino proton region: (A) at room temperature; (B) after 30 min at 38 °C (? = not attributed, x = impurity); (C) after $BPAC_8$ elimination and 24 h at 38 °C (for free decamer 1 = IT2/IT7, 2 = IT10, 3 = IG6, 4 = IG3)

plementary Material for free decamer NMR investigation), and, particularly, the variation of angle ζ (zeta in Table S4 in Supplementary Material) suggest that the oxygen atoms of the central phosphate and in the G3pA4 step are more external with respect to the other oxygen in the decamer backbone and would hence interact more readily with the ligand.

The entity which forms when the temperature is raised to 38 °C differs markedly from the low temperature species in several respects. In contrast to the latter, this species builds up slowly, as seen from the time-dependence of the NMR spectra and of the gel analyses (Fig. 5). Once formed, the molecular association appears to be fairly stable. It resists dissociation, after two precipitation–redissolution cycles, and has a well-defined stoichiometry under the conditions of electrophoresis (in the absence of excess ligand), and of MALDI spectrometry. The slow rate of its appearance and its stability strongly suggest that this association corresponds now to the formation of covalent adducts. This hypothesis is fully confirmed by the examination of the exchangeable proton region in the 1D and 2D NMR spectra, where ligand-modified guanine amino protons, otherwise not visible because of fast rotational broadening and fast exchange, become observable. This shows that a reaction mainly occurs at the amino group of guanine G6 in the central CpG step, G6(N2) [and to a lesser extent at G3(N2)] [Fig. 3(B), boxed area]. Binding of BPA is strongly reminiscent of the reaction of the antibiotic anthramycin with a duplex oligonucleotide where appearance of the guanine amino resonance in the 1D spectra has been previously considered as definitive evidence for formation of a covalent adduct at the exocyclic amino nitrogen of a guanine.[12] That $BPAC_8$ does not become attached to the purine ring itself (in contrast to many other antibiotics where nitrogen N7 of guanine is the addition site) is further confirmed by the absence of significant alteration of ring aromaticity as inferred from the occurrence of only a slight shift in the aromatic H8G6 resonance.[12b]

Retardation of the electrophoretic migration of the adduct,

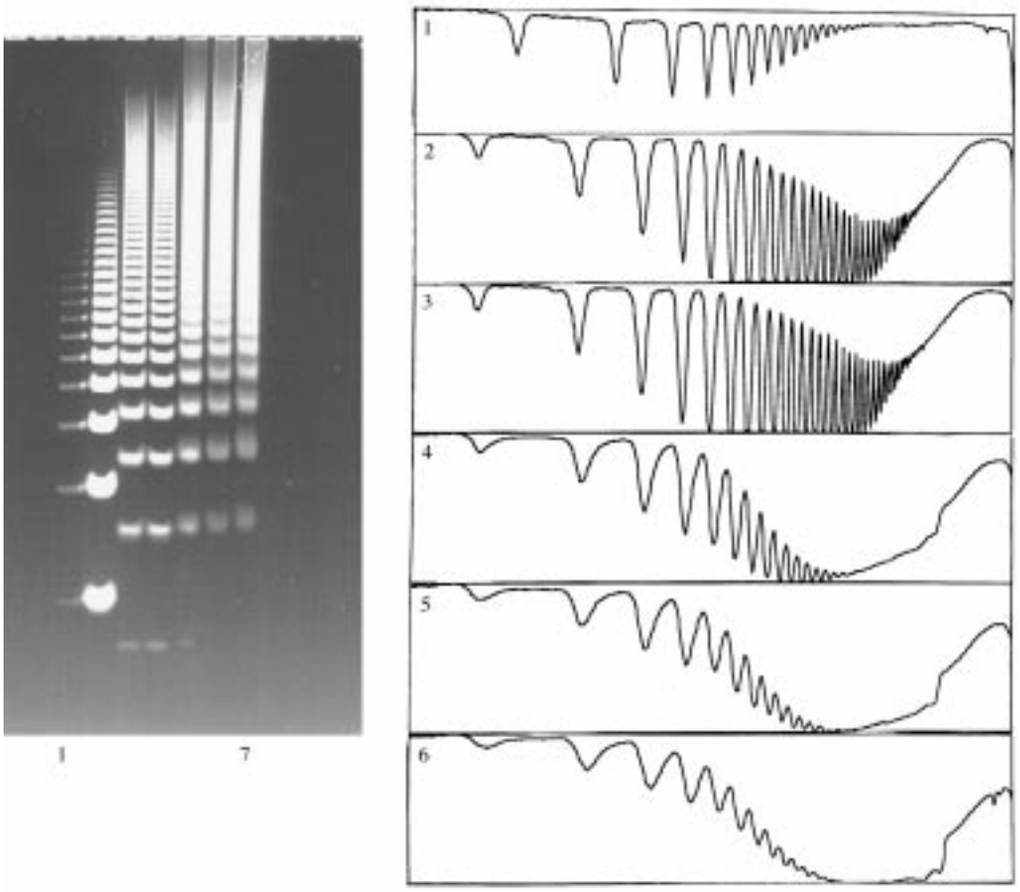
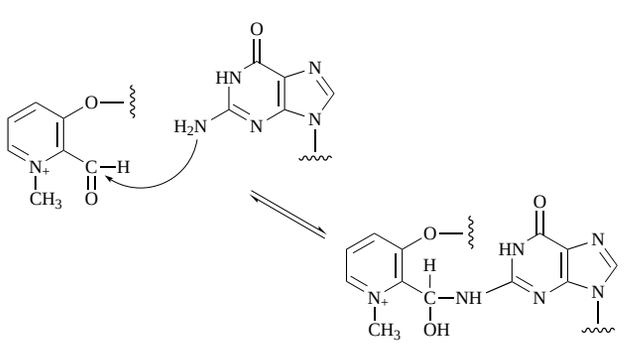


Fig. 5 Interaction of polymerized CRE with $BPAC_8$ at variable time t; $T = 38$ °C; $r = [BPAC_8]/[\text{nucleotide}]$; lanes 1 and 2, control dodecanucleotide; lane 3, polyCRE, $r = 0$; lane 4, $r = 5$, $t = 0$ h; lane 5, $r = 5$, $t = 2$ h; lane 6, $r = 5$, $t = 5$ h; lane 7, $r = 5$, $t = 20$ h. The relative migration distance of each free oligomer n, R_n (with $R_1 = 1$), lane 3, is fitted to its mass M_n by the equation: $M_n = -1508 - 3868\ R_n + 10\,479\ R_n^2 + 1097\ R_n^3$. The equation is subsequently used to evaluate the mass of $BPAC_8$-attached oligomers.

satisfactorily accounted for only by a mass increase corresponding to addition of one ligand molecule per duplex (1 : 1 adduct), indicates no significant bending of the CRE oligomers by $BPAC_8$. NMR results are consistent with this 1 : 1 drug/DNA ratio since duplication of resonances (from NOESY data recorded in D_2O) due to the loss of two-fold helical symmetry establishes the covalent binding to one strand of the duplex.

Another specific feature of the adducts is that they are formed reversibly. Reversible covalent binding is commonly observed in the reaction of aldehydes with amino groups, as in the binding of formaldehyde with exocyclic NH_2 of purines,[13] to lead, in a first step, to a reversible aminal. We propose therefore that the reaction of $BPAC_8$ with the guanine proceeds according to Scheme 2. All these observations are fully consistent with the conclusions from the study on the interaction of BPAs with pBr322 plasmid DNA.[1]



Scheme 2

In short, the overall reaction of $BPAC_8$ with CRE may proceed as follows. In a first step, the dicationic entity would interact with CRE through electrostatic forces, mainly at the central CpG, to lead to an outside-bound complex. When the temperature is now raised to 38 °C, the amino group of guanine G6 becomes susceptible to reaction with the carbonyl groups. Preferential binding at G6 may result from several factors: (i) as at low temperature (see above), the ligand is already favorably positioned in the ionic complex; (ii) consistent with our starting hypothesis, the CpG step presents significant structural distortions with respect to a regular B-DNA[3] which induces a local flexibility (this latter being even more marked when temperature is increased) and a subsequent enhanced reaction rate at this site; (iii) binding at the amino group of G6 in the minor groove (and not at C5 amino in the major groove) may result from marked negative electrostatic potential in this groove which has become large enough to accommodate the dication. Finally, to account for the slight interaction at G3 observed in the NMR spectra, one may postulate that, together with the main adducts where only one aldehyde moiety of $BPAC_8$ is bound to one strand in the duplex, a low population stemming from either single binding at G3 or double binding at G6 and G3 exists.

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