Site-Specific d(GpG) Intrastrand Cross-Links Formed by Dinuclear Platinum Complexes. Bending and NMR Studies†

J. Kašpárková,‡ K. J. Mellish,§ Y. Qu,§ V. Brabec,‡ and N. Farrell*,§

Institute of Biophysics, Academy of Sciences, Brno, Czech Republic 61265, and Department of Chemistry, Virginia Commonwealth University, Richmond, Virginia 23284-2006

Received May 15, 1996; Revised Manuscript Received October 1, 1996⊗

ABSTRACT: The novel platinum drugs [{*trans*-PtCl$(NH_3)_2$}$_2$$H_2N(CH_2)_nNH_2$]$^{2+}$ (1,1/t,t) are currently undergoing preclinical development. The bifunctional DNA binding of these agents allows comparison with that of cisplatin [Farrell et al. (1995) *Biochemistry, 34,* 15480]. The major DNA lesion of cisplatin, the 1,2-d(GpG) intrastrand adduct, produces a rigid, directed bend 30−35° into the major groove of DNA. We have now completed a structural analysis of the corresponding adduct formed with the dinuclear complexes. Gel retardation assays on 15−22 bp oligonucleotides containing a central d(TG*G*T) site show that the (Pt,Pt)-intrastrand adducts result in a flexible nondirectional bend. This bend is essentially independent of chain length ($n = 2, 4, 6$). Chemical reactivity assays indicated a hypersensitivity of the thymine 5′ to the adduct and an enhanced sensitivity of the 3′-thymine to OsO_4. 2D ^{1}H NMR studies on a d(TG_1G_2T) adduct of [{*trans*-PtCl$(NH_3)_2$}$_2$$H_2N(CH_2)_6NH_2$]$^{2+}$ have delineated the structural features responsible for these observations. In contrast to the cisplatin adduct, which displays a 100% N-type sugar of the 5′-G and an *anti* base conformation of the platinated bases in both solid state and solution, the dinuclear adduct does not display the typical N-type sugar pucker. The base orientations are *anti* (5′-T), *anti* (G_1), *anti/syn* (G_2), and *anti* (3′-T) while the sugar conformations are N, S/N, N, and S, respectively. The 5′-T remains stacked with its guanine neighbor while the 3′-T becomes unstacked, a reverse of the situation observed for *cis*-DDP.

DNA is an appealing target for antitumor drugs because the sequence specificity, topological constraints, and localized, even transient, conformations involved in gene regulation are all properties capable of modification by binding of small molecules. Among the alterations of its secondary and tertiary structure to which DNA may be subject, the role of intrinsic bending of DNA is increasingly recognized as of potential importance in regulating transcription and replication functions through specific DNA−protein interactions. Cisplatin (*cis*-[$PtCl_2(NH_3)_2$], *cis*-DDP) is an example of a clinically very important anticancer drug which induces DNA bending upon Pt-DNA adduct formation. The bending of site-specifically modified intrastrand (Pil & Lippard, 1992) or interstrand (Kašpárková & Brabec, 1995) cross-links is recognized by proteins containing the HMG-domain motif. For *cis*-DDP adducts, the structural details responsible for bending and subsequent protein recognition have recently been elucidated by a solid-state X-ray structure determination on a d(CCTCTG*G*TCTCC)-d(GGAGACAGAGG) dodecamer containing one intrastrand cross-link (Takahara et al., 1995). Bending of DNA has further been confirmed by solution ^{1}H NMR spectroscopy studies on a d(CCTG*G*TCC)-d(GGACCAGG) octamer containing the *cis*-DDP intrastrand cross-link (Yang et al., 1995). Notably, the details contributing to the bending in a site-specific interstrand cross-link formed between guanines of two adjacent base pairs have been reported (Huang et al., 1995). The fact that the structural modifications caused to DNA by the antitumor inactive isomer, *trans*-DDP, are not recognized by HMG-domain proteins implicates some specific consequences of the *cis*-DDP-DNA structural modification as responsible for the dramatic difference in cytotoxicity and antitumor activity between the two isomers. The details of these consequences are still not resolved—possibilities such as shielding of the adducts from repair proteins, diversion of damage-recognition proteins by *cis*-DDP-DNA adducts, or the possibility that this recognition is the first step in repair have all been suggested and are capable of examination through choice of suitable model systems (Lippard, 1993).

Our synthetic work on new platinum derivatives has been driven by the hypothesis that platinum drugs which bind to DNA in a fundamentally different manner to that of *cis*-DDP will have altered biological properties including spectrum and intensity of antitumor activity (Farrell, 1995, 1996b). Lending cogency to this argument is the consensus that direct structural analogs of *cis*-DDP do not display a vastly different spectrum of antitumor activity (Christian, 1992; Dorr & Noel, 1993). The relative efficiency of the biological action of analogs (as indicated by assays measuring cell death or by monitoring DNA replication and transcription) is dependent on the kinetics of adduct formation as well as the specific structure of the Pt-DNA adduct formed. Carboplatin differs from *cis*-DDP only in the more inert dicarboxylato leaving group but forms the same bifunctional adducts (Blommaert et al., 1995). All *cis*-DDP analogs with different carrier amines such as 1,2-diaminocyclohexane or

† The authors acknowledge support of this work by American Cancer Society Grant DHP-2E, Boehringer Mannheim Italia, and the Grant Agency of the Czech Republic (Grants No. 307/96/0996 and 301/95/1264). The research of V.B. was also supported in part by an International Research Scholar's award from the Howard Hughes Medical Institute.

‡ Institute of Biophysics.

§ Department of Chemistry, VCU.

⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1996.

S0006-2960(96)01160-9 CCC: $12.00 © 1996 American Chemical Society

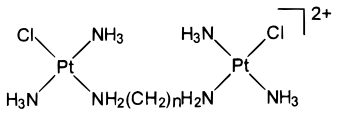
cyclohexylamine produce an array of adducts very similar to those of *cis*-DDP, and it is therefore not surprising that they induce similar biological consequences (see for example, Page et al., 1990; Hartwig & Lippard, 1992).

Platinum coordination compounds display a vast potential for systematic variation of DNA-binding properties which, coupled with appropriate pharmacokinetic properties, affords the promise for rational drug design and more specific inhibition of critical cellular and biochemical targets. We are currently exploring the relationship between the antitumor activity and DNA binding of dinuclear platinum complexes which are undergoing preclinical evaluation (Farrell, 1995, 1996a). Dinuclear platinum complexes with two monofunctional coordination spheres (see Figure 1) are capable of intrastrand cross-link formation (in analogy with cisplatin, platination of two adjacent G bases on the same DNA strand) and interstrand cross-link formation (each Pt moiety binds to opposite strands of the DNA) (Farrell et al., 1995; Farrell, 1995). The (Pt,Pt) intrastrand cross-link is thus the direct analog of the major *cis*-DDP adduct. Given the recent advances in our understanding of the structural basis for the bending of DNA caused by *cis*-DDP, it is of considerable interest to examine how the structure of a dinuclear adduct modified at exactly the same site affects conformational properties such as bending. Since, as stated, bending and subsequent protein recognition are responsible for as yet undefined "downstream" effects, it is important to examine how these properties may be modified. In this paper we report on studies on the bending of site-specific (Pt,Pt) intrastrand cross-links in oligonucleotides. The results of a solution ^{1}H NMR study of a single-stranded tetranucleotide representing the central -TGGT- sequence common to the oligonucleotides studied are also presented to delineate the structural features responsible for the bending.

MATERIALS AND METHODS

Chemicals. [{*trans*-$[PtCl(NH_3)_2\}_2H_2N(CH_2)_nNH_2]Cl_2$, n = 2,4,6 (bisPt-2,[1] bisPt-4, and bisPt-6, respectively), were prepared as described previously (Farrell et al., 1990). The diaqua species [{*trans*-$[Pt(H_2O)(NH_3)_2\}_2H_2N(CH_2)_nNH_2]^{4+}$ (Qu and Farrell, 1990) were generated from bisPt-2, bisPt-4, and bisPt-6 (0.5 mM) by the addition of 1.9 mol equiv of $AgNO_3$ in 10 mM $NaClO_4$ at 37 °C for 24 h in the dark. The AgCl precipitate was removed by centrifugation. All enzymes used in this work were from Bethesda Research Laboratories. Osmium tetroxide (OsO_4) and diethyl pyrocarbonate (DEPC) were from Sigma. Chloroacetaldehyde (CAA) from Fluka was doubly distilled before use. Acrylamide, bis(acrylamide), urea, and NaCN were from Merck.

Oligonucleotides and Their Platinations. The oligodeoxyribonucleotides (Figure 1) were synthesized, purified, allowed to react with the platinum compounds, and repurified as described previously (Brabec et al., 1992). Briefly, the oligonucleotides synthesized on an Applied Biosystems solid-phase synthesizer were purified by ion-exchange FPLC. The



BisPt-n (n = 2, 4, 6)

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d(TGGT)/d(ACCA) (15):   5' CTCCTTGGTCTCCTT
                            AGGAACCAGAGGAAG 5'

d(TGGT)/d(ACCA) (19):   5' CCTCTCCTTGGTCTCCTTC
                            GAGAGGAACCAGAGGAAGG 5'

d(TGGT)/d(ACCA) (20):   5' CCTCTCCTTGGTCTCCTTCT
                            GAGAGGAACCAGAGGAAGAG 5'

d(TGGT)/d(ACCA) (21):   5' CCTCTCCTTGGTCTCCTTCTC
                            GAGAGGAACCAGAGGAAGAGG 5'

d(TGGT)/d(ACCA) (22):   5' CCTCTCCTTGGTCTCCTTCTCT
                            GAGAGGAACCAGAGGAAGAGAG 5'
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FIGURE 1: Structure of bisPt-*n* (n = 2, 4, 6), and sequences of the synthetic oligodeoxyribonucleotides used in the present study with their abbreviations. The top and bottom strands of each pair are designated top and bottom, respectively, in the text. The bold letters in top strands indicate the location of the intrastrand cross-link after modification of the oligonucleotides by bisPt complexes in the way described in the experimental section.

single-stranded oligonucleotides (the top strands in Figure 1) were reacted in stoichiometric amounts with diaqua derivatives of bisPt-2, bisPt-4, or bisPt-6. The platinated oligonucleotides were purified by FPLC. It was verified by platinum flameless atomic absorption spectrophotometry and by the measurements of the optical density that the modified oligonucleotides contained two platinum atoms. It was also verified using dimethyl sulfate (DMS) footprinting of platinum on DNA (Lemaire at al., 1991; Brabec & Leng, 1993) that in the platinated top strands the N7 position of both neighboring guanines was not accessible for reaction with DMS. Briefly, platinated and unmodified top strands (5′-end-labeled with ^{32}P) were reacted with DMS. DMS methylates the N7 position of guanine residues in DNA, producing alkali labile sites (Maxam & Gilbert, 1977). However, if N7 is covalently bound to platinum, it cannot be methylated. The oligonucleotides were then treated with hot piperidine and analyzed by denaturing polyacrylamide gel electrophoresis. For the unmodified oligonucleotides, shortened fragments due to the cleavage of the strand at the two methylated guanine residues were observed in the gel. However, no such bands were detected for the platinated oligonucleotides. These results indicate that one bisPt molecule was coordinated to neighboring guanine residues, forming the 1,2-d(GG) intrastrand cross-link. The platinated strands were allowed to anneal with unplatinated complementary strands (the bottom strands in Figure 1) in 50 mM NaCl plus TE buffer, pH 7.4.

Ligation and Electrophoresis. Unplatinated single strands (bottom strands in Figure 1) were 5′-end-labeled with [γ-^{32}P]-ATP by using T4 polynucleotide kinase. Then they were annealed with their phosphorylated complementary strands [unplatinated or containing 1,2-d(GG) intrastrand cross-links of bisPt-*n*]. Unplatinated and intrastrand cross-link containing duplexes were allowed to react with T4 DNA ligase. The resulting samples along with ligated unplatinated duplexes were subsequently examined on 8% native polyacrylamide [mono:bis(acrylamide) ratio = 29:1] electrophoresis gels. Other details of these experiments were as described in previously published papers (Bellon & Lippard, 1990; Koo et al., 1986).

[1] Abbreviations: bisPt-*n*: [{*trans*-$[PtCl(NH_3)_2\}_2H_2N(CH_2)_nNH_2]Cl_2$ (n = 2, 4, 6); CAA: chloroacetaldehyde; DEPC: diethyl pyrocarbonate; DMS: dimethyl sulfate; DSS: 5,5-dimethylsilapentanesulfonate; dqf-COSY: double quantum filtered correlated spectroscopy; HMQC: ^{1}H detected heteronuclear multiple quantum coherence correlated spectroscopy; NOESY: nuclear Overhauser effect spectroscopy; TE buffer: 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4; TMP: trimethyl phosphate; TOCSY: total correlated spectroscopy.

Chemical Modifications. The top or bottom strand was 5′-end-labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase before they were annealed with their complementary nonlabeled strands. The modifications of double-stranded oligonucleotides by OsO_4, DEPC, and CAA were performed as described previously (Marrot & Leng, 1989) with minor alterations. The modification by OsO_4 was performed in the presence of 2,2′-bipyridine; both OsO_4 and 2,2′-bipyridyl were at a concentration of 0.75 mM, and the reaction was performed for 10 min at 20 °C. The modification by CAA was performed in 0.1 M sodium acetate, pH 6.4, for 1 h at 25 °C; the concentration of CAA was ca. 0.3 M. In the case of the platinated oligonucleotides, platinum was removed after reaction of the DNA with the probe by incubation with 0.2 M NaCN (alkaline pH) at 45 °C for 10 h in the dark.

NMR Spectroscopy. The reaction of bisPt-4 or bisPt-6 (4 mM in 99.996% D_2O) with 1 equiv of d(TGGT) was followed at 37 °C for 24 h by ^{1}H NMR spectroscopy; spectra were recorded on a Varian Gemini 300 MHz spectrometer with DSS (5,5-dimethylsilapentanesulfonate) as an internal standard. d(TGGT) was synthesized with an Expedite 8909 DNA synthesizer (Perseptive Biosystems) using the cyanoethyl phosphoramidite method (reagents were from Glen Research, Sterling, VA). In addition, bisPt-6 was dissolved in deionized H_2O at a concentration of 50 μM and 1 equiv of d(TGGT) added (unadjusted pH = 5.5). The solution was kept at 37 °C for 3 days and the solvent then removed by rotary evaporation. The reaction product was lyophilized twice from 1 mL of D_2O and then dissolved in 99.999% D_2O at a concentration of approximately 4 mM for 2D NMR analysis. ^{1}H−^{1}H dqf-COSY (Rance et al., 1983), TOCSY (Braunschweiler et al., 1983), NOESY (Jeener et al., 1979), and ^{1}H−^{31}P HMQC (Keniry, 1996) experiments were performed at 28 °C on a Varian Unity Plus spectrometer at a ^{1}H frequency of 500 MHz and a ^{31}P frequency of 202 MHz. Data were collected using the States method (States et al., 1982). The following parameters were used for data acquisition: dqf-COSY: 512 complex increments in t_1, each with 960 complex points in t_2, sweep width 7486 Hz, 64 transients per t_1 increment, 1 s relaxation delay between transients; TOCSY: MLEV-17 pulse sequence, 300 complex t_1 increments, 1024 complex t_2 points, sweep width 7486 Hz, 64 transients, relaxation delay 1.1 s, mixing time 0.8 s; NOESY: 254 complex t_1 increments, 1024 complex t_2 points, sweep width 7486 Hz, 64 transients, relaxation delay 1.6 s, mixing time 0.4 s; HMQC: 100 complex t_1 increments (^{31}P dimension), 1024 complex t_2 points (^{1}H dimension), sweep width 7486 Hz (t_2) and 5000 Hz (t_1), 256 transients, relaxation delay 1 s. ^{31}P spectra were referenced to external TMP (trimethyl phosphate, 1 M in D_2O). The data were processed on a Silicon Graphics Indigo2 workstation using Felix 2.3. The data were apodized with a 90° phase-shifted sine-bell window function in both dimensions and zero-filled to 2048 (^{1}H) or 512 (^{31}P) points prior to Fourier transformation.

RESULTS

Gel Electrophoresis. The top strands of the oligonucleotide duplexes examined in the present work were designed to contain only one high-affinity platinum binding site, the two adjacent guanine bases of the intrastrand cross-link (Figures 1 and 2). All sequences were designed to leave a 1 bp overhang at their 5′-ends in double-stranded form. These overhangs facilitate polymerization of the monomeric oligonucleotide duplexes by T4 DNA ligase in only one orientation, and maintain a constant interadduct distance throughout the resulting multimer. Autoradiograms of electrophoresis gels revealing resolution of the ligation products of 15, 19−22 bp duplexes d(TGGT)/d(ACCA)(15,-19−22) (Figure 1) containing a unique 1,2-d(GG) intrastrand

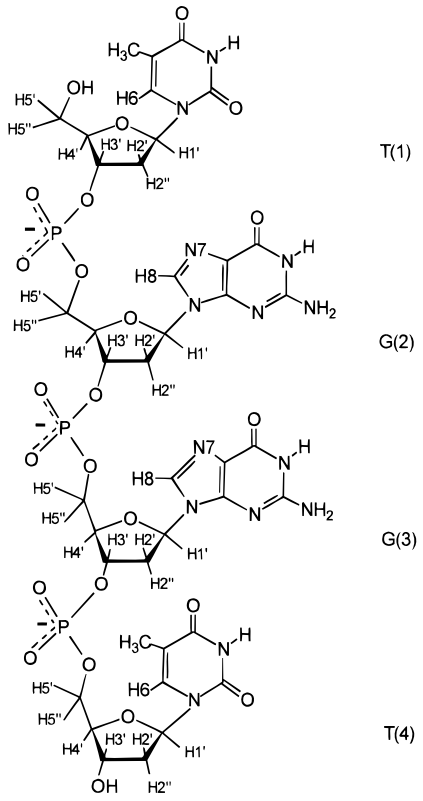

FIGURE 2: Structure and numbering scheme of the central d(TGGT) sequence of oligodeoxyribonucleotides examined.

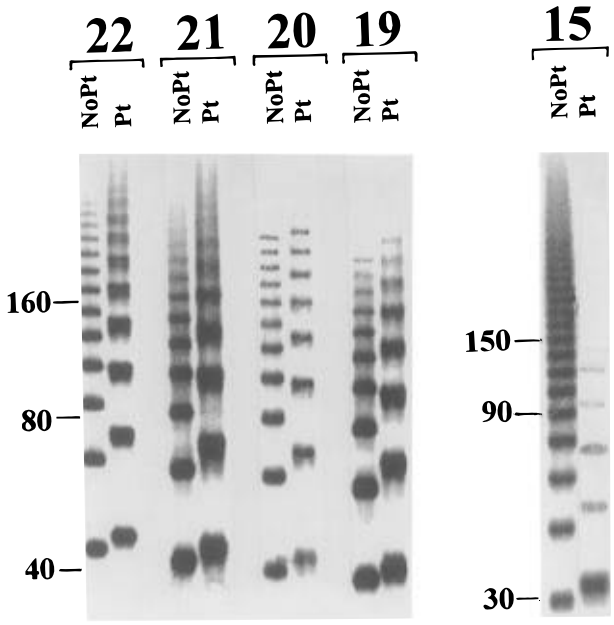

FIGURE 3: Autoradiograms of the ligation products of double-stranded oligonucleotides d(TGGT)/d(ACCA)(15,19−22) containing a unique bisPt-4 intrastrand cross-link separated on an 8% polyacrylamide gel. Unplatinated oligomers, lanes No Pt; platinated oligomers, lanes Pt.

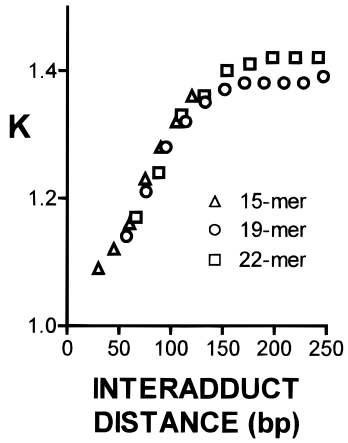

FIGURE 4: Plot showing the relative mobility *K versus* sequence length curves for the oligomers d(TGGT)/d(ACCA)(15,19,22), denoted respectively as 15-mer, 19-mer, and 22-mer.

cross-link of bisPt-2, -4, or -6, are shown in Figure 3. A significant retardation was observed for the multimers of all platinated duplexes. Decreased gel electrophoretic mobility may result from a decrease in the DNA end-to-end distance (Lerman & Frisch, 1982; Lumpkin & Zimm, 1982). Various platinum(II) complexes have been shown to form DNA adducts which decrease gel mobility of DNA fragments due to either stable curvature of the helix axis or increased isotropic flexibility (Rice et al., 1988; Bellon & Lippard, 1990; Leng, 1990; Bellon et al., 1991; Brabec et al., 1993; Malinge et al., 1995). DNA multimers of identical length and number of stable bend units, but with differently phased bends, have different end-to-end distances. The DNA bends of a multimer must be, therefore, spaced evenly and phased with the DNA helical repeat in order to add constructively. Such constructively phased bends add in plane, yielding short end-to-end distances and the most retarded gel migration. In other words, gel electrophoresis of multimers of oligonucleotide duplexes which only differ in length and contain a stable curvature induced by the same platinum adduct should exhibit a phase effect, i.e. the maximum retardation should be observed for the multimers having the bends in phase with the helix screw. In contrast, the normal electrophoretic mobility should be observed for the multimers with bends separated by a half-integral number of DNA turns. Importantly, a gel mobility retardation of multimers due to the platinum adducts introducing isotropic flexibility rather than stable curvature is not expected to display a phase dependence (Leng, 1990). The *K* factor is defined as the ratio of calculated to actual length. The calculated length is based on a multimer's mobility, and is obtained from a calibration curve constructed from the mobilities of unplatinated multimers. The variations of the *K* factor *versus* sequence length obtained for the multimers of the duplexes 15, 19, and 22 bp long and containing the unique 1,2-d(GG) intrastrand cross-link of bisPt-4 are shown in Figure 4. The same plots obtained for the multimers of the duplexes 20 and 21 bp long and containing the intrastrand cross-link (not shown) were superimposable on those shown in Figure 4. Thus, no significant change in the slopes of these plots was observed in a broad range of the lengths of the monomeric duplexes (15−22 bp). Similar results were obtained (not shown) for the multimers of the duplexes containing 1,2-d(GG) intrastrand cross-link of bisPt-2 or bisPt-6. These results suggest that conformational distortion induced in

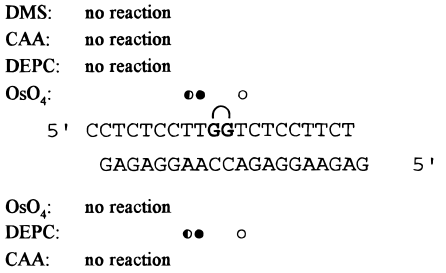

FIGURE 5: Summary of the reactivity of chemical probes. ●, ◐, and ○ designate strong, medium, or weak reactivity, respectively.

double-stranded DNA by intrastrand cross-links formed between neighboring guanine residues by dinuclear platinum compounds does not result in a stable curvature (directional bending). The results are consistent with the view that the (Pt,Pt)-d(GpG) lesion, caused by monofunctional Pt binding to two adjacent guanines, increases flexibility of the duplex.

Chemical Probes of DNA Conformation. In order to further characterize the distortion induced in DNA by (Pt,-Pt)-d(GG) intrastrand cross-links, the d(TGGT)/d(ACCA)-(20) containing the 1,2-d(GG) intrastrand cross-link of bisPt-2,4 or 6 was treated with several chemical agents that are used as tools for indicating the existence of conformations other than canonical B-DNA. These agents include OsO_4, DEPC, and CAA, which react preferentially with single-stranded DNA and distorted double-stranded DNA (Nielsen, 1990). The results obtained with chemical probes are summarized in Figure 5. In all cases, the pattern and degree of reactivity toward the chemical probes were essentially identical for all dinuclear complexes, independent of chain length of the diamine linker.

OsO_4 is hyperreactive with thymine residues and slightly reactive with cytosines in single-stranded nucleic acids and in distorted DNA as compared to B-DNA (Palecek et al., 1990). OsO_4 reacted with no residue within the unplatinated duplex (Figure 6, left, lane ds). Mainly thymine residues were reactive strongly in the unplatinated single-stranded top oligonucleotide (Figure 6, left, lane ss). The duplexes containing the (Pt,Pt) intrastrand cross-link showed strong reactivity of the two neighboring 5′-thymine residues in the top strand adjacent to the adduct (shown for bisPt-4 in Figure 6, lane dsPt). A weak but significant reactivity was also observed for the first 3′-thymine residue adjacent to the cross-link. No reactivity between OsO_4 and the residues in the bottom strand of the cross-linked duplex was apparent.

DEPC carbetoxylates purines at the N(7) position. It is hyperreactive with unpaired and distorted adenine residues in DNA and with left-handed Z-DNA (Herr, 1985; Johnston & Rich, 1985). Adenine and guanine residues within the unplatinated single-stranded oligonucleotides (top and bottom) readily reacted with DEPC (shown for the bottom strand in Figure 6, right, lane ss). However, the adenines adjacent to the (Pt,Pt) cross-link in the *single-stranded* top oligomer were no longer modified (not shown). Similarly, no reactivity of adenine and guanine residues was observed within the unplatinated double-stranded oligonucleotide (shown in Figure 6, right for the bottom strand, lane ds). Within the double-stranded oligonucleotide containing the (Pt,Pt)-d(GG) intrastrand cross-link, three base residues in the bottom strand became reactive (Figure 6, right, lane dsPt): these are readily identified as the three adenine residues complementary to the reactive thymine residues of the top strand.

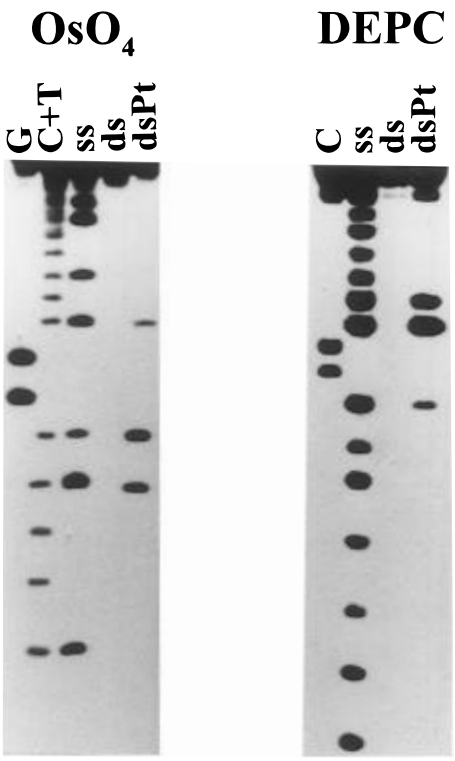

FIGURE 6: Piperidine-induced specific strand cleavage at OsO_4-modified (left) and diethyl pyrocarbonate-modified (right) bases in unplatinated and platinated d(TGGT)/d(ACCA)(20). The oligomers were 5′-end-labeled at their top strands in the case of the modification by OsO_4 or at their bottom strands in the case of the modification by DEPC. OsO_4: The lanes G and C+T are Maxam−Gilbert specific reactions for the unplatinated duplex that had only top strand end labeled. The lane ss is relative to the unplatinated top strand. The lane ds is relative to the unplatinated duplex. The lane dsPt is relative to the duplex containing bisPt-4 intrastrand cross-link. DEPC: The lane C is Maxam−Gilbert specific reaction for the unplatinated duplex that had only bottom strand end labeled. The lane ss is relative to the unplatinated bottom strand. The lane ds is relative to the unplatinated duplex. The lane dsPt is relative to the duplex containing bisPt-4 intrastrand cross-link.

CAA reacts with N(1) and N(6) of adenine and N(3) and N(4) of cytosine residues. It is hyperreactive with these residues in single-stranded DNA, which makes this probe particularly suitable for observing base residues in denatured regions of DNA. CAA is also hyperreactive with adenine and cytosine residues in the junctions between B- and Z-DNA and with adenine residues in Z-DNA as compared with B-DNA (Kohwi-Shigematsu et al., 1987; Lilley, 1983; McLean et al., 1987). All adenine and cytosine residues within the unplatinated top and bottom single strands readily reacted with CAA (not shown). No reactivity of these residues with CAA was, however, observed within the unplatinated duplexes or duplexes containing the (Pt,Pt)-d(GG) intrastrand cross-link.

NMR Spectroscopy. General Features. The reactions of bisPt-4 and bisPt-6 with d(TGGT) (1:1 ratio) were monitored by ^{1}H NMR spectroscopy at 37 °C. The d(TGGT) sequence represents the central sequence in all of the oligonucleotides studied for bending. The downfield 5−8 ppm region of the ^{1}H NMR spectrum of d(TGGT) in D_2O consists of four singlets for each of the nonexchangeable base protons (two G H8 and two T H6 signals) and four triplets for each of the sugar H1′ protons (Figure 7A). Assignment of peaks (Figure 7A, Table 1) followed that of Spellmeyer Fouts et al. (1988). The changes in spectra upon addition of the

Table 1: ^{1}H NMR Parameters for d(TGGT) and Its Adducts with Bis-Platinum Drugs (37 °C)

	δ H8 (ppm)	δ H6 (ppm)	δ H1′ (ppm)
d(TGGT)	7.95, 7.85	7.53, 7.40	6.21, 6.11, 6.04, 5.89
d(TGGT)/bisPt-4[a]	8.70, 8.63	7.69, 7.56	6.32, 6.32, 6.06, 6.06
d(TGGT)/bisPt-6[b]	8.62, 8.60	7.71, 7.57	6.32, 6.27, 6.07, 6.07

[a] pH 6.7. [b] pH 6.4.

Table 2: ^{1}H Chemical Shift Assignments (ppm) for the d(TGGT)/BisPt-6 Adduct (28 °C, pH 8.8)[a]

	H8/H6	CH_3	H1′	H2′	H2″	H3′	H4′	H5′/H5″
5′ T(1)	7.67	1.93	6.10	1.89	2.37	4.90	4.19	3.93, 3.81
G(2)	8.41		6.28	2.37	2.37	4.56	4.13	3.82, 3.82
G(3)	8.35		6.20	2.91	2.79	5.09	4.36	4.06, 3.73
3′ T(4)	7.49	1.89	6.08	1.94	2.18	4.36	3.98	4.05, 3.62

[a] H2′ and H2″ were assigned according to the strength of their NOEs to H1′: the H1′−H2″ cross-peak is more intense than the H1′−H2′ cross-peak (Wijmenga et al., 1993).

dinuclear platinum compounds paralleled those observed for the analogous study with the dinucleotide d(GpG) (Qu et al., 1996). Four new H8 signals were observed corresponding to the two monofunctionally platinated intermediates (i.e. one platinum bound at 5′G or 3′G with the corresponding 3′G/5′G still uncomplexed). The intermediate signals gradually disappeared, to be replaced by two H8 signals corresponding to the 1,2-d(GG) intrastrand cross-link, which became the major end product within 12h. Other minor signals were observed which may correspond to cross-links between two d(TGGT) molecules or nonspecific polymer formation. Table 1 shows the assignment of the chemical shifts for the G H8, T H6, and sugar H1′ protons of the adducts. For both bisPt-4 and bisPt-6, the H8 signals were shifted 0.7−0.8 ppm downfield (indicative of N7 binding) compared to free d(TGGT). The small difference in chemical shift between the two H8 protons (<0.1 ppm in both cases) is nevertheless greater for the $n = 4$ than the $n = 6$ derivative (21 Hz *versus* 6 Hz, respectively). This is in good agreement with the observations on the d(GpG) system, indicating that, in both systems, the H8 protons are in near chemical equivalence, characteristic of GG adducts lacking steric strain (den Hartog et al., 1985). The two H6 signals were shifted only slightly downfield from d(TGGT) (0.1−0.2 ppm), and the four H1′ protons now appeared as overlapping multiplets between 6.4 and 6.0 ppm.

Structural Determination. The bisPt-6/d(TGGT) adduct was chosen for a more detailed structural examination and for comparison with that of the corresponding d(GpG) adduct characterized previously (Qu et al., 1996). The conformation was examined using a combination of through-space nuclear Overhauser effect (NOESY) and through-bond *J* correlated (dqf-COSY, TOCSY, HMQC) 2D NMR spectroscopy. The nonexchangeable protons of the adduct were assigned as follows: the four sets of sugar protons and the methylene groups of the bisPt-6 diamine chain were distinguished by dqf-COSY and TOCSY; the coupling between thymine H6 and CH_3 was detected by TOCSY, and the base protons were assigned to their respective sugar rings by intranucleotide NOEs; the 3′-thymine residue was identified by the absence of coupling between H3′ and ^{31}P; and the sequential order of bases was determined by internucleotide NOEs.

Table 2 shows the chemical shifts of the base and sugar protons of the d(TGGT)/bisPt-6 adduct. The base and H1′

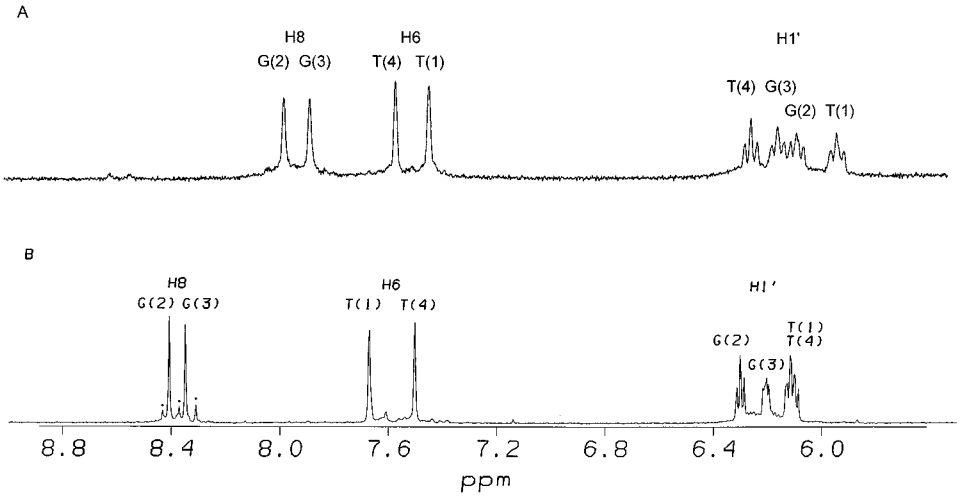

FIGURE 7: 1D ^{1}H NMR Spectra in D_2O of the base and H1′ region of (A) d(TGGT) and (B) d(TGGT)/bisPt-6 adduct. Assignments for d(TGGT) based on Spellmeyer Fouts et al. (1988). * represents polymer side products.

region of the 1D ^{1}H NMR spectrum is shown in Figure 7B. For the bisPt-6 adduct, the H8 signal of G(2) is downfield from that of G(3). The opposite is observed for single-stranded 1,2-d(GG) intrastrand adducts of *cis*-DDP (Kozelka et al., 1992). Also, a large downfield shift of the G(3) H8 is observed for the adducts of *cis*-DDP and related compounds with d(TGGT); e.g., *cis*-DDP: G(2) ΔH8 0.3 ppm, G(3) ΔH8 1.2 ppm (Spellmeyer Fouts et al., 1988). Three ^{31}P resonances were identified by HMQC at −3.82 ppm (TpG), −2.90 ppm (GpG), and −3.68 ppm (GpT). Such a downfield shift of the GpG phosphate is characteristic of 1,2-d(GG) intrastrand platinum adducts (Spellmeyer Fouts et al., 1988). Upon coordination of bisPt to chiral oligonucleotides such as d(GpG) and d(TGGT), the CH_2 signals of the diamine linker become inequivalent. For the corresponding bisPt-6/d(GpG) adduct, all six possible signals were observed (Qu et al., 1996). Analysis of the TOCSY and COSY data set of the d(TGGT) adduct showed four multiplets for a total of six methylene groups of the 1,6-$NH_2(CH_2)_6NH_2$ diamine chain: δ 2.72 (both H_2NCH_2), δ 2.15 ($H_2NCH_2CH_2$), δ 2.05 ($H_2NCH_2CH_2$), and δ 1.60 ppm (central CH_2CH_2). The signals were broad, indicating that some restricted rotation must occur.

The full structural analysis of the d(TGGT) adduct is more complicated than for d(GpG) because of extensive overlap of chemical shifts. The H1′ protons of G(2) and G(3) appear as well separated multiplets (Figure 7B). In principle, the relative proportions of N (C3′-endo) and S (C2′-endo) type sugar may be obtained by detailed analysis of the individual sugar coupling constants (Rinkel & Altona, 1987; Orbons et al., 1986). The coincidence of δ(H2′) and δ(H2") chemical shifts, (Table 2) made it impossible to resolve $^3J_{H1'-H2'}$ and $^3J_{H1'-H2''}$ for the present resolution. Nevertheless, the lack of the characteristic doublet for the 100% N-type sugar of the 5′G in *cis*-DDP adducts ($^3J_{H1'-H2'} < 1$ Hz) is striking. This feature implies a greater contribution of the S-type conformation to the shape of the multiplet. The sum of the coupling constants, $\Sigma^3J_{H1'}$, measured as the distance between the outer peaks of the multiplet, is 12.80 and 10.17 Hz for G(2) and G(3), respectively. For free d(TGGT), $\Sigma^3J_{H1'}$ was approximately 14 Hz for all four sugar residues, indicating predominantly (>70%) S-type conformation (Rinkel & Altona, 1987). These results are taken to indicate a high % S conformation for the G(2) sugar and a lower S/N ratio for G(3) of the bisPt-6 d(TGGT) adduct. At high expansion, the multiplet for the H1′ proton of the G(3) sugar appears as a quartet, allowing tentative assignment of $^3J_{H1'-H2'}$ and $^3J_{H1'-H2''}$ values of 3.61 and 6.56 Hz, respectively. Now, the distinctly lower value for $^3J_{H1'-H2'}$ is strong support for a predominant N-type conformation on this sugar (Rinkel & Altona, 1987). Further information on the sugar conformations of the (Pt,Pt)-d(TGGT) adduct can be obtained from the cross-peaks between H1′ and H2′/H2″ in the dqf-COSY spectrum (Figure 8). No coupling was observed between H1′ and H2′ of T(1), indicative of a 100% N-type conformation of the sugar ring. For G(2) the overlap of H2′ and H2″ chemical shifts results in one intense cross-peak

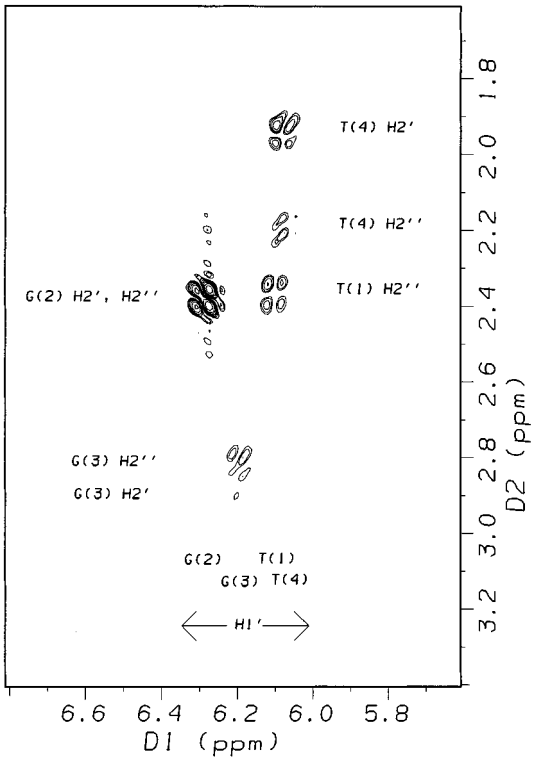

FIGURE 8: H1′ and H2′/H2″ region of the dqf-COSY spectrum of the d(TGGT)/bisPt-6 adduct (28 °C, pH 8.8 in D_2O). Note the absence of coupling between T(1) H1′ and T(1) H2′. Coupling is observed between T(1) H1′ and T(1) H2″, G(2) H1′ and G(2) H2′ and H2″, G(3) H1′ and G(3) H2′ (very weak) and H2″, and T(4) H1′ and T(4) H2′ and H2″ (weak).

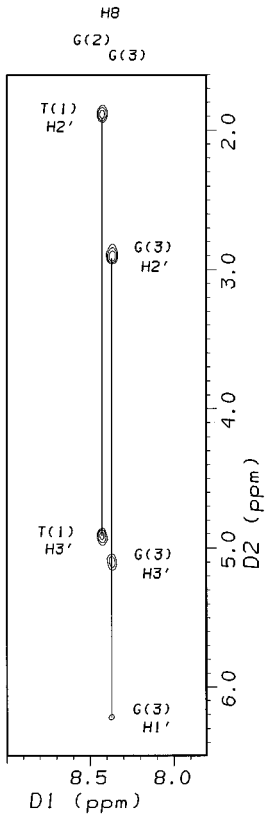

FIGURE 9: NOESY spectrum of the d(TGGT)/bisPt-6 adduct (28 °C, pH 8.8, in D_2O) showing cross-peaks between G(2) H8 and T(1) H3′ and H2′, and G(3) H8 and G(3) H1′, H3′, and H2″.

with H1′. The weak coupling between H1′ and H2′ of G(3) is also apparent. H1′ of T(4) was coupled to both H2′ and H2″; the weaker coupling to H2″ is indicative of >70% S-type conformation (Wijmenga et al., 1993). The spectral resolution did not permit a detailed interpretation and a more quantitative estimate of the N/S ratio, but for the present purposes this is not strictly necessary as the qualitative differences with *cis*-DDP adducts are clear. For the case of d(TGGT) with [Pt(en)Cl_2], predominantly S-type conformation was observed for all residues except for the 5′-G(2), which was shown to be in the 100% N-type conformation (Spellmeyer Fouts et al., 1988).

The region of the NOESY spectrum of the d(TGGT)/bisPt-6 adduct showing NOEs between H8 and sugar protons is shown in Figure 9. Internucleotide NOEs were observed between H8 of G(2) and H2′ and H3′ of T(1), indicating base stacking between T(1) and G(2). No other internucleotide NOEs were observed. For the [Pt(en)Cl_2]-d(TGGT) adduct, base stacking was instead observed between G(3) and T(4) as evidenced by an NOE between T(4) H6 and G(3) H2″ (Spellmeyer Fouts et al., 1988). An *anti* base orientation is suggested for both T(1) and G(2) by the lack of an NOE between H8 or H6 and the relevant H1′ (Patel et al., 1982). A strong NOE was observed between G(3) H8 and both G(3) H2′ (characteristic of *anti* base orientation and S-type sugar) and G(3) H3′ (characteristic of *anti* base orientation and N-type sugar) (Wijmenga et al., 1993). In addition, a weak NOE was observed between G(3) H8 and G(3) H1′, indicative of *syn* base orientation (Patel et al., 1982). Therefore, it appears that G(3) is part *anti*, part *syn*. This is supported by the relatively downfield H2′ resonance of G(3) at 2.91ppm; H2′ protons of nucleotides with *syn* base orientation have previously been observed at 3.1−3.7 ppm (Mao et al., 1995; Norman et al., 1989; Yang et al., 1995). The existence of *syn/anti* equilibria has been noted previously for the *cis*-DDP adduct of d(GCG) (den Hartog et al., 1983). For T(4) there was a strong NOE between H6 and H2′, showing *anti* base orientation and confirming S-type sugar conformation.

Table 3: Base and Sugar Conformation of the Adducts of [Pt(en)Cl_2][a] or BisPt-6 with d(TGGT)

	sugar conformation		base orientation	
	[Pt(en)Cl_2]	bisPt-6	[Pt(en)Cl_2]	bisPt-6
5′ T(1)	S	N	*anti*	*anti*
G(2)	N	S/N	*anti*	*anti*
G(3)	S	majority N	*anti*	*anti*/*syn*
3′ T(4)	S	S	*anti*	*anti*

[a] From Spellmeyer Fouts et al. (1988).

DISCUSSION

This paper describes the conformational distortion induced in DNA by the (Pt,Pt) intrastrand cross-link between neighboring guanine residues. The bending experiments were carried out with the double-stranded oligodeoxyribonucleotides (15−22 bp) containing the unique intrastrand adduct in their central sequence d(TGGT)/d(ACCA) (Figure 1). The phasing assay revealed that the (Pt,Pt) intrastrand cross-link does not result in a stable curvature (directional bending). Lack of phase dependence of the retardation of gel mobility of DNA containing the 1,2-d(GG) intrastrand cross-links was consistent with the view that the dinuclear platinum adduct increases flexibility of the duplex. An increased flexibility introduced to the helix in this manner is sustained by the observation that this lesion creates a local conformational distortion revealed by the chemical probes (Figures 5 and 6). This distortion occurs on both sides of the cross-link and extends mainly over five bp. The lack of reactivity of CAA with the platinated duplex indicates that the (Pt,Pt) intrastrand cross-link does not create single-stranded regions in DNA (Kohwi-Shigematsu et al., 1987; Lilley, 1983; McLean et al., 1987). Thus, the reactivity of the thymine residues cannot be ascribed to the presence of single-stranded regions. Importantly, the increased flexibility of the duplex and the extent of distortion characterized by chemical probes are independent of the length of the diamine bridge linking the two Pt units.

Some structural features responsible for the difference in bending caused by mononuclear and dinuclear adducts were elucidated by the NMR experiments. The structure of the d(TGGT)/bisPt-6 adduct is compared with *cis*-DDP analogs in Table 3. The major differences are (i) the absence in the dinuclear adduct of the 100% N-type sugar of the 5′-guanine compared to the *cis*-DDP adduct; (ii) the *syn/anti* equilibrium around the 3′-guanine, previously unobserved for *cis*-DDP adducts; and (iii) in the dinuclear adduct the 5′-thymine remains stacked with the neighboring guanine. In contrast, no evidence of *syn* base orientation was observed for the adduct of d(TGGT) with [Pt(en)Cl_2] (Spellmeyer Fouts et al., 1988). Further, the 5′-thymine was unstacked because of the steric constraints of bifunctional binding of the one Pt atom to the two adjacent guanines (Spellmeyer Fouts et al., 1988). This feature has also been observed in the recent crystal structure of the *cis*-DDP adducted dodecamer (Takahara et al., 1995).

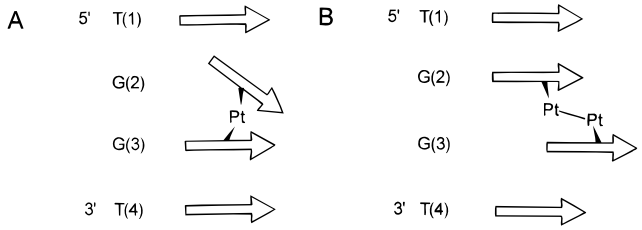

FIGURE 10: Schematic representation of the structures of the adducts of d(TGGT) with (A) cisplatin and (B) bisPt-6.

The configuration of the two guanine bases in the bisPt-6/d(GpG) adduct is best indicated as "stepped head-to-head" without any major reorientation of either of the guanine bases (Qu et al., 1996). This situation is in contrast to that for *cis*-DDP, where the steric demands of bifunctional binding of *cis*-DDP to two adjacent guanines contribute to a large dihedral angle between the nucleobases ("head-to-head" configuration), eventually reflected in the rigid directed bend in DNA (Takahara et al., 1995; Sherman et al., 1988; Admiraal et al., 1987; Yang et al., 1995). The conformational features of the "normal" *cis*-DDP adduct of d(GG) sequences are a 100% N-type 5′-sugar observed within an *anti*−*anti* base conformation. In the *cis*-DDP adduct of d(TGGT), there is continued stacking of the 3′-T but destacking of the 5′-T leading toward the observed bend (Spellmeyer Fouts et al., 1988). The lack of a directed bend observed in the bending studies reported here is a reflection of the inherently more flexible dinuclear adduct. In marked contrast to the situation for *cis*-DDP, the principal structural features derived for both the d(GpG) (Qu et al., 1996) and d(TGGT) adducts of bisPt-6 are the presence of a *syn* base conformation and a predominantly N-type sugar for the 3′-guanine, and a mixed N/S conformation of the 5′-sugar. In d(TGGT) the "stepped head-to-head" model with a *syn* G(3) allows for continued stacking of the 5′-T but destacking of the 3′-T, in perfect accordance with the NMR data (Figure 10). For the dinuclear adducts, the results mean that a bulky adduct with no pronounced bending is produced. Thus, the adduct may present a block to DNA polymerase (Zou et al., 1994) but may not be a substrate for recognition by HMG-domain proteins which require bending as a recognition motif. Thus, the concept of *differential* protein recognition within identical sequences based on subtle changes in adduct structure appears plausible.

ACKNOWLEDGMENT

The authors would like to thank Dr. Neel Scarsdale for help with the measurement of 2D NMR spectra.

SUPPORTING INFORMATION AVAILABLE

One figure showing the full 1D ^{1}H NMR spectrum of the d(TGGT)/bisPt-6 adduct (1 page). Ordering information is given on any current masthead page.

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BI961160J