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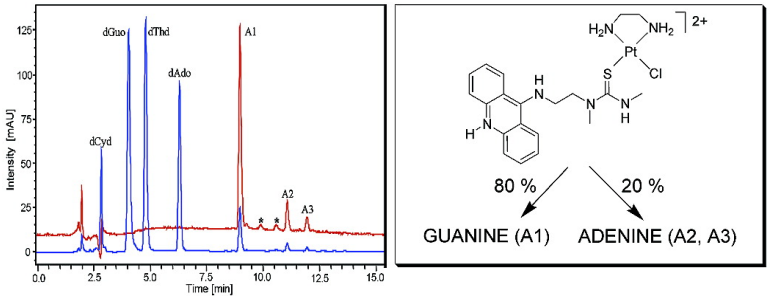

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Unprecedented Monofunctional Metalation of Adenine Nucleobase in Guanine- and Thymine-Containing Dinucleotide Sequences by a Cytotoxic Platinum−Acridine Hybrid Agent

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Abstract: We have investigated the reactions of [PtCl(en)(ACRAMTU-*S*)]$(NO_3)_2$ (**2**) (en = ethane-1,2-diamine; ACRAMTU = 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea, acridinium cation, **1**), the prototype of a new class of cytotoxic DNA-targeted agents, with 2′-deoxyguanosine (dGuo) and random-sequence native DNA by in-line liquid chromatography/mass spectrometry (LC/MS) and NMR spectroscopy (^{1}H, ^{195}Pt) to identify the covalent adducts formed by platinum. In the mononucleoside model system, two adducts are observed, [Pt(en)(ACRAMTU)(dGuo)]$^{3+}$ (**P1**, major) and [Pt(en)(dGuo)$_2$]$^{2+}$ (**P2**, minor). The reaction, which proceeds significantly slower (half-life 11−12 h at 37 °C, pH 6.5) than analogous reactions with cisplatin and reactions of **2** with double-stranded DNA, results in the unexpected displacement of the sulfur-bound acridine ligand in ∼15% of the adducts. This reactivity is not observed in double-stranded DNA, rendering **1** a typical nonleaving group in reactions with this potential biological target. In enzymatic digests of calf thymus DNA treated with **2**, three adducts were identified: [Pt(en)(ACRAMTU)(dGuo)]$^{3+}$ (**A1**, ∼80%), [Pt(en)(ACRAMTU){d(GpA)}]$^{2+}$ (**A2**, ∼12%), and [Pt(en)(ACRAMTU){d(TpA)}]$^{2+}$ (**A3**, ∼8%). **A1** and **P1** proved to be identical species. In the dinucleotide adducts **A2** and **A3**, complex **2** covalently modifies adenine at GA and TA base steps, which are high-affinity intercalation sites of the acridine derivative **1**. **A2** and **A3**, which may be formed in the minor groove of DNA, are the first examples of monofunctional adenine adducts of divalent platinum formed in double-stranded DNA. The analysis of the adduct profile indicates that the sequence specificity of **1** plays an important role in the molecular recognition between DNA and the corresponding conjugate, **2**. Possible biological consequences of the unusual adduct profile are discussed.

Introduction

The design of sequence- and groove-specific DNA-targeted molecules plays an important role in postgenomic drug discovery. Numerous cancer therapeutics produce their cytotoxic effect by damaging nuclear DNA, thereby interfering with chromosome replication and gene-regulatory processes of the cell. Structurally novel pharmacophores that produce new types of DNA adducts may trigger events in cell metabolism that ultimately lead to (apoptotic) cancer cell death.[1] Synthetic metal-containing compounds that cause cellular damage at the DNA level, such as *cis*-diamminedichloroplatinum(II) (cisplatin), have demonstrated clinical utility in cancer chemotherapy.[2]

The coordination chemistry and many aspects of the molecular biology of cisplatin are now well understood and have been the subject of detailed reviews.[3,4] Briefly, the nucleophilic attack of guanine-N7 in the major groove is the first binding step in the formation of all known cisplatin−DNA adducts. Subsequent closure of the monofunctional adducts to bifunctional cross-links may then occur with guanine-N7 or adenine-N7. The well-characterized 1,2-intrastrand cross-link between neighboring purines,[5] a lesion that is recognized by minor-groove binding proteins,[6] has been implicated as a possible mediator of the cytotoxic effect of cisplatin. The binding of *cis*-[PtCl(H_2O)-$(NH_3)_2$]$^+$, the activated form of cisplatin, to DNA is driven by simple electrostatic interactions,[7] and adduct formation occurs without noticeable long-range sequence specificity.[8] The high selectivity of divalent platinum for guanine-N7 in DNA is common to all second- and third-generation cisplatin analogues as well as nonclassical agents, such as polynuclear complexes[9] and complexes exhibiting trans geometry.[10] Several approaches

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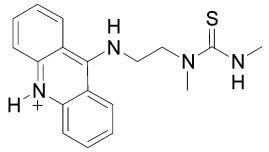
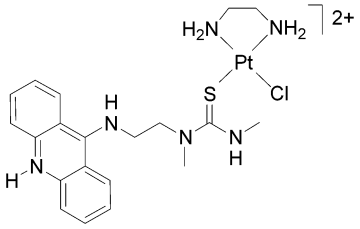
Chart 1. Structures of the Novel 9-Aminoacridine Derivative and Its Platinum Conjugate



1 (ACRAMTU)



2 (PT-ACRAMTU)

attempting to produce platinum drugs with altered DNA-binding profiles by attaching the metal to DNA intercalators or groove binders have been reported. In virtually all cisplatin−intercalator complexes, however, the *cis*-diaminedichloroplatinum(II) moiety dominates the sequence specificity of the conjugates, leading to 1,2-intrastrand cross-links in runs of adjacent guanine bases.[11] Characteristically, even the tethering of platinum to suitably modified distamycin, a minor-groove binding agent with a distinct preference for AT-rich domains in DNA,[12] results in an array of covalent guanine adducts similar to that of cisplatin.[13]

To reduce or eliminate the cisplatin character of platinum−acridine complexes and to produce an alternative antitumor profile, we argue that it is necessary to completely prevent the metal from forming cross-links in G-rich regions of DNA. Toward this objective, we have developed[14] a prototypical cytotoxic platinum−acridine conjugate ("PT−ACRAMTU", **2**) by substituting one chloro leaving group in $[PtCl_2(en)]$ (en = ethane-1,2-diamine), a cisplatin analogue, with sulfur of the novel acridine derivative, 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea ("ACRAMTU", **1**) (Chart 1). In this complex, thiourea sulfur and bidentate amine presumably act as typical nonleaving groups that are not displaced by DNA nucleophiles. This critical feature should not only prevent platinum from cross-linking purines on DNA, but may also lead to the formation of monofunctional covalent adducts that is controlled by the sequence and groove specificity of the intercalating unit rather than the metal moiety.

In a recent study[15] we employed various physicochemical and biophysical methods to shed light on the DNA modifications of **1**, **2**, and related compounds in cell-free systems. Conjugate **2** unwinds negatively supercoiled pUC19 plasmid by 21°/adduct, which indicates (partial) intercalation of acridine into the DNA base stack and confirms the persistence of the sulfur-mediated platinum−acridine linkage in covalent DNA adducts of **2**.[15] This type of "pseudobifunctional" mode of binding was first proposed for *cis*-$[Pt(NH_3)_2$(9-aminoacridine)$Cl]^+$ and related complexes.[16] The acridinium cation **1** itself binds strongly to native DNA ($pK_a \approx 9.8$;[14] $K_i = 1.5 \times 10^6\ M^{-1}$) and exhibits a 1.6-fold preference for AT over GC in alternating synthetic copolymers.[15] Using 2-D NMR and molecular modeling techniques we were able to unequivocally establish the site(s) of intercalation of the acridine moiety and the groove specificity of the noninter-calating thiourea side chain in **1**.[17] In self-complementary model duplexes, **1** selectively intercalated into the TA/TA, CG/CG, and GA/TC base steps with the thiourea residue located in the minor groove (2-D NOESY experiments), as predicted from previous[15] physical data.

We have now studied the covalent interactions of the corresponding platinum conjugate, **2**, both in a simple mono-nucleoside model system and in random-sequence native DNA using NMR spectroscopy and liquid chromatography/mass spectrometry (LC/MS). The unusual findings presented in this contribution strongly suggest that the intercalator unit in complex **2** alters the binding-site specificity of divalent platinum, leading to covalent adenine adducts, possibly in the minor groove of DNA. While guanine adducts are still the predominant lesion, the high frequency of adenine binding (approximately 20% of the DNA adducts identified) clearly renders **2** a non-guanine specific platinum-based DNA modifier.

Experimental Section

Materials. PT−ACRAMTU (**2**, nitrate salt) was synthesized according to the published procedure.[14] Stock solutions of **2** in the appropriate buffers were prepared immediately prior to the incubations and, if necessary, stored at −20 °C. 2′-Deoxyguanosine (dGuo) and calf thymus DNA were purchased from Sigma. The mononucleoside was used as supplied. The hexamer, 5′-GAGAGA-3′, was synthesized using phosphoramidite chemistry and desalted prior to incubation with platinum. Calf thymus DNA was dissolved in 20 mM Tris buffer (pH 7.1) and purified by dialysis against buffer using a dialysis tube with a 10 000 Da molecular mass cutoff. The purity of the DNA was assessed spectrophotometrically from the ratio of absorbances at 260 and 280 nm giving a value of 1.8, characteristic of RNA- and protein-free DNA.[18] The synthetic alternating copolymer, poly(dA-dT)$_2$ (Pharmacia Amersham Biotech) was used as supplied. The nucleic acids were annealed by slow cooling of the buffered solutions from 80 °C to room temperature to ensure that the DNA was in its double-stranded form. DNA concentrations (base pairs, bp) were determined from absorbances at 260 nm using Beer's law with ϵ_{260} (calf thymus) = 12 824 M^{-1} cm^{-1} (bp) and ϵ_{260}(AT) = 13 100 M^{-1} cm^{-1} (bp).[19] Stock solutions of DNA were stored at −20 °C. Millipore water was used for the

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preparation of all buffers. DNase I, nuclease P1, and alkaline phosphatase enzymes were obtained from Sigma or Amersham. HPLC grade solvents were used in all chromatographic separations. All other (bio)-chemicals (DNase-free, where available) and solvents were purchased from common vendors and used without further purification. UV−visible spectra were recorded on a Hewlett-Packard 8453 spectrophotometer.

Reactions. Reactions between **2** and dGuo were carried out under various conditions at 30−40 °C in water for 72−96 h. The pH of the unbuffered solutions was adjusted to 6−7 immediately after combining equimolar amounts of the reactants (10−50 mM). A maximum pH drop of 0.8 units was observed during incubations. The progress of the reaction was followed by reverse phase high-performance liquid chromatography (HPLC). The chromatographic separations of the adduct mixtures were carried out by analytical and semipreparative HPLC or ion-exchange chromatography as described below. Calf thymus DNA and poly(dA-dT)$_2$ ($c_{DNA} = 10^{-3}$ M (bp); 20 mM Tris buffer, pH 7.1) were incubated with **2** at platinum-to-nucleotide ratios (r_i) of 0.2, 0.05, and 0.01 at 37 °C for 18 h.

Enzymatic Digestion. The following protocol was used to digest 1000-μL samples of the platinum-modified DNAs (total incubation time 26 h at 37 °C): (i) + 200 μL of 50 mM $MnCl_2$ + 200 units DNase I (2 h); (ii) + 130 units DNase I (2 h); (iii) + 80 units nuclease P1 (2 h); (iv) + 20 units nuclease P1 (16 h); (v) + 100 units alkaline phosphatase + 100 μL alkaline phosphatase buffer (500 mM Tris buffer, 10 mM $MgCl_2$, pH 9) (2 h); (vi) + 60 units alkaline phosphatase (2 h). The mixtures were centrifuged at 13 000 rpm for 5 min, and the supernatant was collected. The digested samples were desalted against water for 6 h at 4 °C using disposable dialysis tubes with a 100 Da (±10%) molecular mass cutoff (Spectrum Labs) and stored at −20 °C.

Chromatographic Separations. HPLC separations of the product mixtures resulting from incubations of **2** with dGuo were performed on a LaChrom Hitachi D-7000 system equipped with a L-7420 UV−visible variable-wavelength detector. For analytical purposes, aliquots of the reaction mixtures were collected, appropriately diluted, and immediately loaded on the HPLC column. Reactions were monitored at 254 nm, and pure fractions of the adducts were collected using Hamilton PRP-1 reverse-phase columns (250 × 4.6 mm/7 μm for analysis, flow rate 1.5 mL/min; 250 mm × 10 mm/10 μm for isolation of adducts, flow rate 5.0 mL/min). *Eluents*: solvent A = 84% 0.1 M NH_4OAc/12% MeOH/4% MeCN; solvent B = 60% MeOH/20% MeCN/20% 0.1 M NH_4OAc). *Gradient*: 100% A→100% B over 20 min. HPLC fractions were stored at −20 °C. Adducts for NMR analysis were isolated by cation-exchange chromatography using a 300 mm × 10 mm CM-Sephadex column (carboxymethylethyl derivative of Sephadex) on a Bio-rad BioLogic HR FPLC workstation. *Eluents*: solvent A = water, solvent B = 1 M NaCl. *Gradient*: 100% A → 100% B over 240 min at a flow rate of 1 mL/min. FPLC fractions were exhaustively desalted against deionized water for 12 h at 4 °C in appropriate disposable dialysis tubes and finally lyophilized to dryness. Adducts in desalted mixtures of enzymatically digested DNA were separated on the HPLC module of an Agilent Technologies 1100 LC/MSD Trap system equipped with a multiwavelength diode-array detector and autosampler. *Column*: 150 mm × 4.6 mm reverse-phase Agilent Zorbax SB-C18, T = 25 °C. *Eluents*: solvent A = 0.1% formic acid in water; solvent B = 0.1% formic acid in MeCN. *Gradient*: 95% A/5% B → 73% A/27% B over 22 min at a flow rate of 0.75 mL/min. Elution of unmodified and Pt−acridine-modified DNA fragments was monitored at 254 and 413 nm, respectively. The four unmodified deoxynucleosides were assigned on the basis of the retention times of known standards (Sigma).

Mass Spectrometry. Mass spectra were acquired on an Agilent Technologies 1100 LC/MSD ion trap mass spectrometer equipped with an atmospheric-pressure electrospray ionization source, which has a standard mass range for singly charged ions of 50−2200 *m*/*z*. Adducts either were directly infused into the electrospray source at a rate of 10 μL/min using a syringe pump (adducts **P1** and **P2**) or were infused after passing through the in-line HPLC system (adducts **A1**−**A3**). Solvent evaporation was assisted by a flow of N_2 drying gas (300 °C, 11−15 L/min). Mass spectra were recorded with enhanced scan resolution (5500 m/z s^{-1}) in positive and negative ion modes over a 100−1800 *m*/*z* range. A capillary voltage of ±1.8 kV was applied. Tandem mass spectra (MS/MS) of the product ions of interest were generated by collision-induced dissociation of the trapped precursor ions (He collision gas). The fragmentation amplitudes varied from 0.4 to 1.0 V, and the isolation width was 4.0 *m*/*z*. All data were analyzed using the LC/MSD Trap Control 4.0 data analysis software.

NMR Spectroscopy. ^{1}H NMR (500 MHz) spectra of **P1** and **P2** were recorded on a Bruker DRX-500 instrument equipped with a 5-mm inverse three-channel *Z*-gradient probe and a variable-temperature unit. All spectra were acquired at 25 and 30 °C with a spectral width of 5000 Hz, 64k data points, 32 transients and a recycle delay of 1 s, and processed with Felix 2000 (Molecular Simulations Inc., San Diego) on a Silicon Graphics O2 workstation. One-dimensional data sets were apodized using exponential multiplication and a line broadening of 0.3 Hz. ^{1}H chemical shifts were referenced to the residual HDO signal, which was calibrated against external 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS). Variable-pH ^{1}H NMR spectra of D_2O solutions of **P1** were recorded at 25 °C in the pH range 1−12 and referenced to internal DSS. The pH was adjusted with 0.1 M DCl and NaOD solutions and measured in the NMR tube using a Beckman micro combination electrode. Reported pH* values are uncorrected pH meter readings of the D_2O solutions. ^{195}Pt NMR spectra were recorded at 107.5 MHz. Typical acquisition and processing parameters were the following: spectral width 27 0000 Hz, flip angle 30°, 500-μs recycle delay, 30 000 transients, 30 Hz line broadening. ^{195}Pt chemical shifts were referenced to an external $K_2[PtCl_4]$ solution in D_2O and are reported vs $Na_2[PtCl_6]$.[20] Details of the 2-D COSY and NOESY experiments have been deposited as Supporting Information.

Results

Reactivity of 2 in a Model System. The proposed mode of DNA binding of PT-ACRAMTU (**2**) involves platination of nucleophilic nucleobase nitrogen and intercalation of the planar acridine chromophore into the DNA base stack adjacent to the metal binding site.[15] Here, we have used HPLC/mass spectrometry and NMR spectroscopy to shed light on the covalent interactions of **2** with 2′-deoxyguanosine (dGuo). While this model reaction does not mimic sequence- and groove-specific interactions of **2** with double-stranded DNA, it provides valuable information on the reactivity of the platinum complex and the leaving-group specificity of adduct formation. Furthermore, the platinum−nucleoside model complexes formed proved to be useful reference compounds for the identification of analogous adducts in the enzymatic digests of DNA treated with **2**.

Figure 1 shows the HPLC profiles of samples taken at various incubation times of a reaction mixture containing equimolar amounts of **2** and dGuo. At 37 °C and pH 6.5, the half-life of free dGuo is 11−12 h. Two new species, **P1** (major) and **P2** (minor) were identified in the chromatograms at retention times of 8.6 and 2.6 min, respectively. ^{1}H NMR spectra of the lyophilized mixtures taken in D_2O (Figure S1) confirm the formation of two platinum−nucleoside adducts, on the basis of the presence of two new sets of guanosine H8 and H1′ signals. The data further indicated that a small amount of the bis(acridine) complex, [Pt(en)(ACRAMTU-*S*)$_2$]$^{4+}$,[21] had formed,

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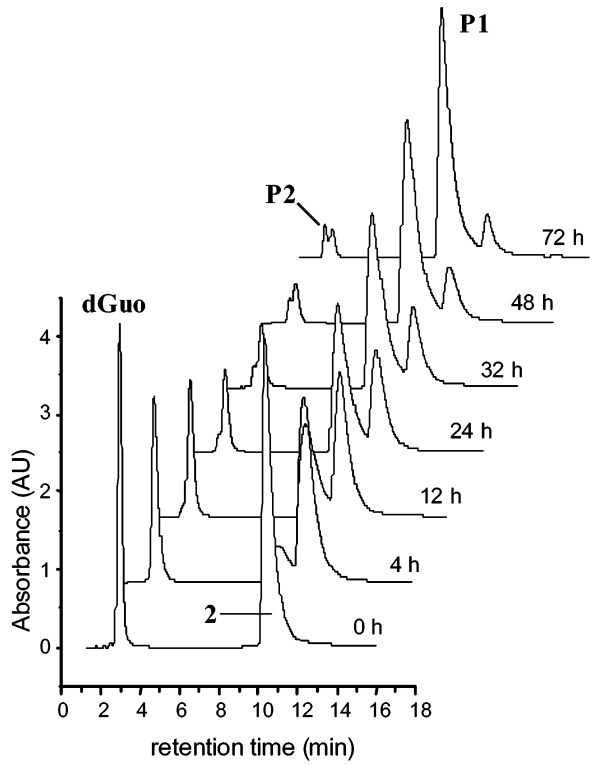


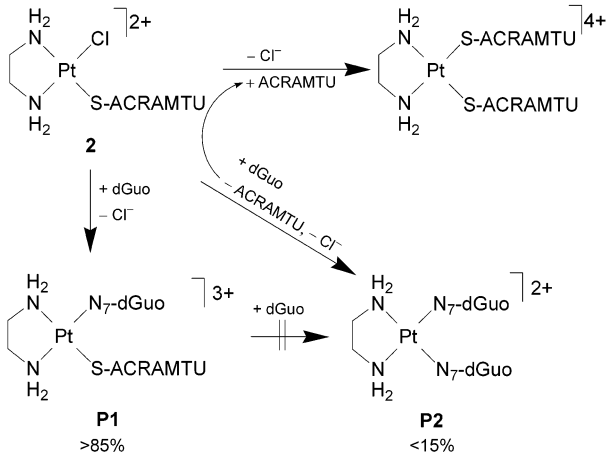
Figure 1. Progress of the reaction between conjugate **2** and dGuo monitored by reverse phase HPLC. The starting materials dGuo and **2** elute at retention times of 3.0 and 10.5 min, respectively. The adducts formed, **P1** (major) and **P2** (minor), are identified at retention times of 8.6 and 2.6 min, respectively. The shoulder present in the base of the **P1** peak is possibly due to the bis(acridine) side product. The monitoring wavelength was 254 nm.

which was supported by the presence of a signal at −3360 ppm in ^{195}Pt NMR spectra. **P1** and **P2** were isolated from these mixtures using semipreparative HPLC and ion-exchange chromatography and analyzed by mass spectrometry and NMR spectroscopy. Despite the relative lability of metal complexes containing DNA constituents in electrospray mass spectrometry,[22] intact ions of all adducts were detected in this study.

P1 was unequivocally identified as the monofunctional adduct $[Pt(en)(ACRAMTU)(dGuo)]^{3+}$ (M_r = 847.3) resulting from replacement of the chloro leaving group with nucleobase nitrogen. The electrospray mass spectrum of **P1** acquired in negative ion mode (Figure 2) shows the $[\mathbf{P1}\text{-}4H]^-$ ion (*m*/*z* 843) and major product ions resulting from loss of en ligand (*m*/*z* 783), loss of dGuo (*m*/*z* 577), and loss of en and dGuo (*m*/*z* 517). Peaks assigned to the platinum-containing ions show the expected Pt isotopic profiles. Other fragments identified in the mass spectrum include free acridine, $[\mathbf{1}\text{-}2H]^-$ (*m*/*z* 323), and free 2′-deoxyguanosine, $[dGuo\text{-}H]^-$ (*m*/*z* 266). Tandem mass spectrometry (Figure S2) confirmed the proposed fragmentation pattern for the $[\mathbf{P1}\text{-}4H]^-$ precursor ion.

^{1}H and ^{195}Pt NMR spectroscopy was used to further elucidate the structure of **P1**. The ^{1}H NMR spectrum of **P1** and signal assignments based on 2-D COSY and ROESY experiments (Table S1, Figure S3) are given in Figure 3. The downfield shift of $\Delta\delta$ 0.47 ppm compared to free dGuo and pH dependence of the guanine-H8 resonance are consistent with platinum

Scheme 1. Reaction of Conjugate **2** with dGuo



binding to the N7 position of the nucleobase.[23] No protonation of guanine was observed around pH 2 (pK_a(N7) ≈ 2.1[24]). Furthermore, a ^{195}Pt NMR chemical shift of δ −2928 was found for this adduct, characteristic of an $N_3S_{thiourea}$ environment[25] of Pt^{2+}.

The minor product, **P2**, was identified as the bis(dGuo) platinum adduct, $[Pt(en)(dGuo)_2]^{2+}$ (M_r = 789.2), resulting from displacement of both the chloro ligand and sulfur-bound ACRAMTU in **2** by guanine-N7. Mass spectrometry and ^{1}H NMR spectroscopic data (Table S1) were used to establish the composition of this adduct. The fragmentation of **P2** due to collisionally activated dissociation (CAD) observed in the positive-ion electrospray mass spectrum (Figure 2) yielded crucial structural information. For $[\mathbf{P2}\text{-}H]^+$ and the other cations studied, CAD generally gave more informative spectra than tandem mass spectrometry of trapped precursor ions.[22] Platinum-containing fragments originating from the $[\mathbf{P2}\text{-}H]^+$ ion (*m*/*z* 788) result from loss of one intact dGuo ligand (*m*/*z* 521), depurination of one (*m*/*z* 671) or both (*m*/*z* 556) nucleosides, and a combination of loss of dGuo and depurination (*m*/*z* 405). The peak at *m*/*z* 152 is assigned to protonated guanine base, $[G + H]^+$. Critical differences are observed in the fragmentation patterns of **P1** and **P2**. The presence of sulfur in the coordination sphere of platinum in **P1** causes trans-labilization and loss of bidentate amine, which is not observed for **P2**. On the other hand, depurination is observed for **P2** but not for **P1**. This is consistent with reduced positive charge on platinated guanine-N7 due to the presence of electron-rich sulfur in **P1**, disfavoring base−sugar glycosidic cleavage.

On the basis of the above analytical and spectroscopic data, the reactions of **2** can be summarized as follows (Scheme 1): The major adduct **P1** (> 85%, NMR) results from substitution of chloride by dGuo, probably via a prehydrolysis pathway.[4] The rate of adduct formation proves to be significantly slower than for simple platinum chloroam(m)ine complexes,[4] possibly due to steric effects of the bulky thiourea group in substitution reactions on the metal center. **P1** does not react with additional dGuo (HPLC). Therefore, the first step in the formation of the minor product **P2** (<15%, NMR) must be substitution of the

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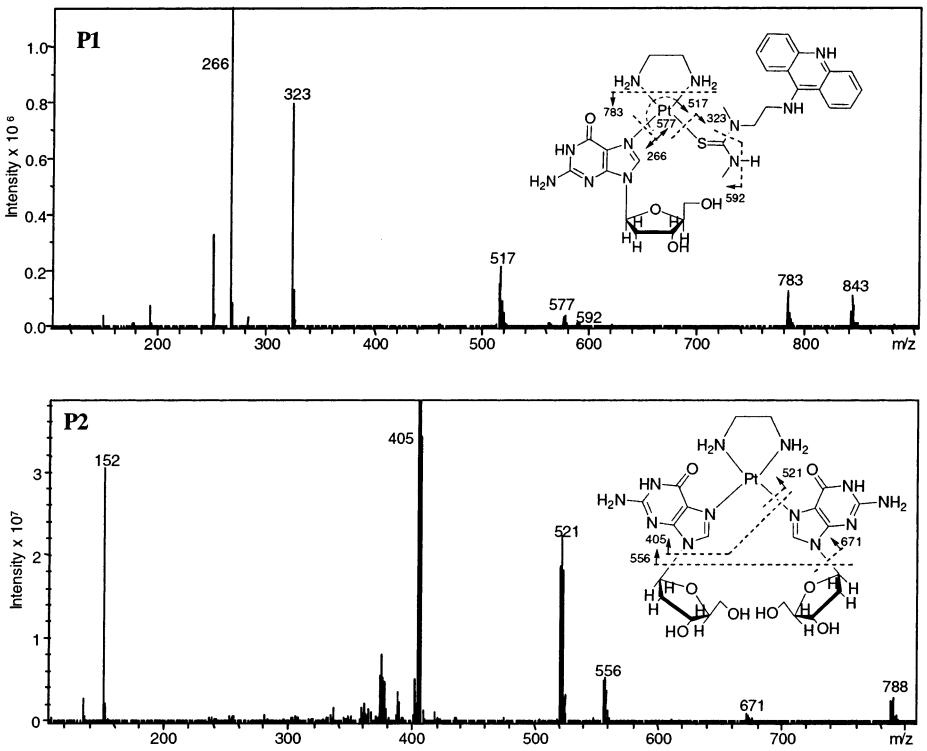


Figure 2. Negative-ion electrospray mass spectrum of adduct **P1** and positive-ion electrospray mass spectrum of adduct **P2** showing peaks for the adducts [**P1**-4H]$^-$ and [**P2**-H]$^+$, respectively, and fragment ions resulting from in-source collisionally activated dissociation (CAD). The insets illustrate the observed fragmentation patterns.

sulfur ligand by dGuo, followed by substitution of chloride by a second equivalent of nucleoside. The reaction of nucleophilic ACRAMTU released in this process with unreacted complex **2** explains the formation of [Pt(en)(ACRAMTU-*S*)$_2$]$^{4+}$.

Reaction of 2 with Native, Random-Sequence DNA. Enzymatic digestion of native DNA covalently modified with **2** and subsequent analysis of the digests by HPLC chromatography/mass spectrometry (in-line LC/MS) was used to establish the nature of the nucleobases involved in platinum binding. Calf thymus DNA was incubated with **2** under physiologically relevant conditions for 18 h (reactions of **2** with DNA proceed considerably faster than reactions with dGuo, and maximum binding levels are usually reached in less than 24 h[15]). Incubations were performed at formal metal-to-nucleotide ratios (r_i) in the range 0.01−0.2. DNase I and nuclease P1 enzymes were used to endonucleolytically digest the DNA samples, and the resulting fragments were dephosphorylated with alkaline phosphatase to afford a mixture of modified and unmodified nucleosides. The desalted digests were then separated by HPLC using gradient eluent systems and reverse phase stationary phases and the separated adducts subjected to in-line mass spectrometry analysis.

Typical HPLC traces obtained for a sample of drug-treated digested DNA are shown in Figure 4. The profile recorded at 254 nm shows baseline-separated peaks assigned to the four natural 2′-deoxynucleosides and additional peaks at retention times of 8.6, 10.8, and 11.8 min. The same chromatogram recorded at a monitoring wavelength of 413 nm, the most intense band in the visible spectrum of the acridine chromophore[15] ($\epsilon \approx 1 \times 10^4$ M^{-1} cm^{-1}), confirms that the peaks labeled **A1**−**A3** represent drug-modified adducts. For **A1**, the most abundant adduct, mass spectra obtained in negative-ion mode (Figure 5) show features identical to those found in spectra of **P1** (see Figure 2), including a peak at *m*/*z* 843 and the corresponding CAD-based fragment ions, suggesting that **A1** and **P1** are identical species. These observations and the fact that a sample of **P1** coelutes with **A1** from the HPLC column provide strong evidence that the major DNA adduct of **2** is the monofunctional guanine adduct, [Pt(en)(ACRAMTU)(dGuo)]$^{3+}$. **A1** was the only detectable platinum−acridine-modified *mononucleoside* in this mixture.

Electrospray mass spectra of **A2** and **A3** acquired in positive-ion mode combined with selective fragmentation by in-source CAD (Figure 5) was used to establish the chemical nature of these adducts. **A2** and **A3** were identified as the platinum-modified guanine-adenine (GA)- and thymine-adenine (TA)-containing *dinucleotide* sequences, [Pt(en)(ACRAMTU){d(GpA)}]$^{2+}$ (M_r = 1159.5) and [Pt(en)(ACRAMTU){d(TpA)}]$^{2+}$ (M_r = 1134.3). Peaks for the intact adducts are observed in both spectra at *m*/*z* 1158 ([**A2**-H]$^+$) and *m*/*z* 1133 ([**A3**-H$^+$]), respectively. The latter ion was also detected in digests of poly(dA-dT)$_2$ treated with **2** (not shown). The mass spectra show fragmentation patterns that are in agreement with platinum binding to the adenine base in both cases. Critical fragment ions are observed, which result from selective depurination (cleavage of the adenine-ribose CN glycosidic bond, giving *m*/*z* 712) and combined depurination/loss of en ligand (*m*/*z* 652), respectively. Both pathways lead to fragments, in which platinum remains attached to adenine. Under the experimental conditions used, another major product ion is observed at *m*/*z* 578, resulting from dissociation of the platinum complex from the adenine nucleobase. In adduct **A3**, minor fragments at *m*/*z* 808 and *m*/*z* 325 are consistent with the dissociation of the Pt−S bond. The proposed sequence specificity of adduct formation in the above

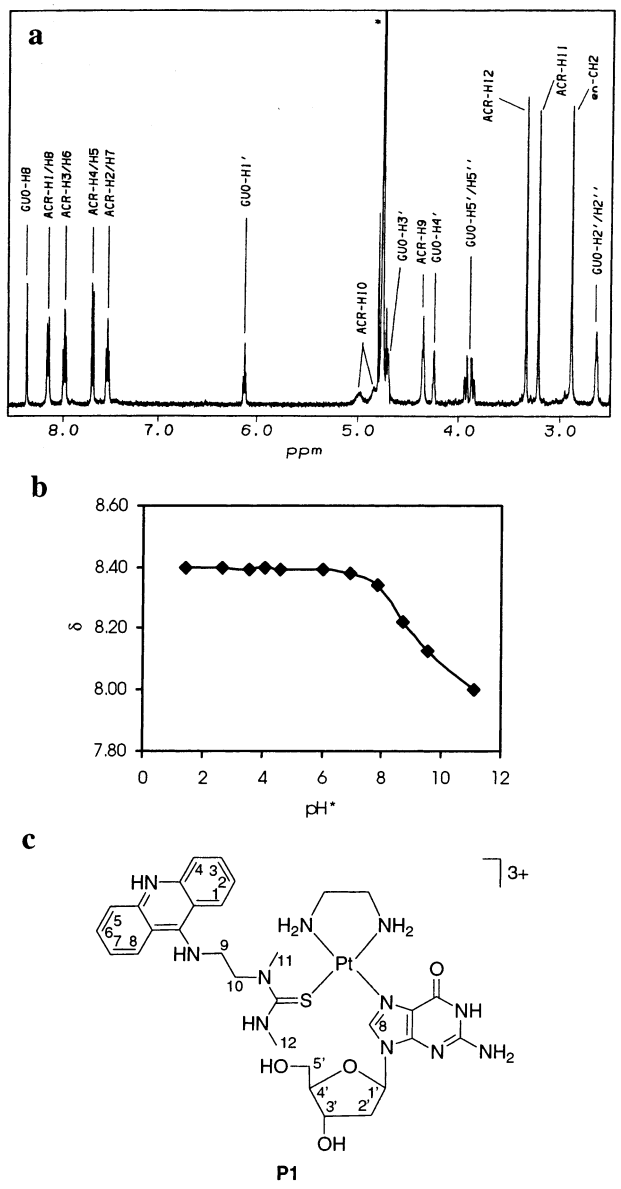


Figure 3. (a) 500 MHz ^{1}H NMR spectrum of adduct **P1** (pH* 6.5, D_2O, 25 °C, $c \approx 4$ mM) giving signal assignments. The asterisk denotes the HDO signal. (b) pH dependence of the guanine-H8 chemical shift in **P1**. (c) Structure of **P1** giving atom numbering for nonexchangeable protons.

dinucleotides, resulting in 5′-GpA*-3′ and 5′-TpA*-3′ modified sequences (rather than 5′-A*pG(T)-3′), is partly based on the fact that no *w*-type nucleotide fragments could be detected in the negative-ion (tandem) mass spectra of these species. Depurination of the 5′ adenine base in 5′-A*pG(T)-3′ would be expected to lead to facile cleavage of the C3′-O bond in the deoxyribose residue of the modified nucleobase (Scheme 2).[26]

The DNA adduct profile of conjugate **2** was established with high reproducibility at the various incubation ratios studied. On the basis of the integration of the peaks in the HPLC profiles recorded at 413 nm, the three adducts, **A1**−**A3**, form at a ratio of approximately 80:12:8. Characteristically, no bifunctional adducts $[Pt(en)(dGuo)_2]^{2+}$ and $[Pt(en)\{d(GpG)\}]^+$, which would signal the formation of inter- and intrastrand cross-links,

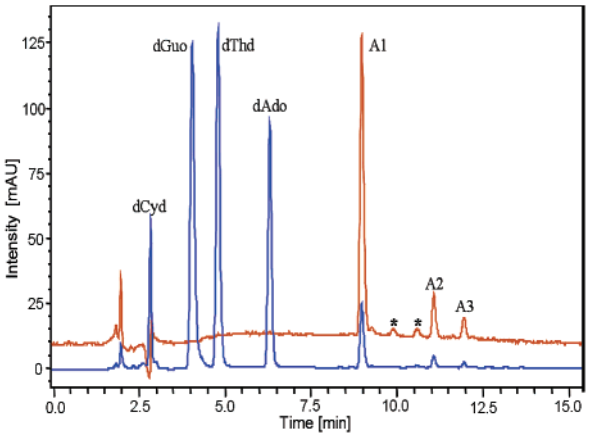
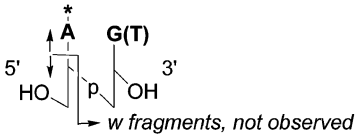


Figure 4. Reverse-phase HPLC elution profiles at monitoring wavelengths of 254 nm (bottom trace) and 413 nm (top trace) of an enzymatic digest of calf thymus DNA treated with conjugate **2** at $r_i = 0.05$. Unmodified mononucleosides elute at retention times <7 min. The Pt−acridine-modified DNA fragments elute at 8.6 min (**A1**), 10.8 min (**A2**), and 11.8 min (**A3**). The asterisks indicate minor unidentified adducts.

Scheme 2. Formation of *w* Fragments Expected for 5′ Depurination



respectively[27] (promoted by the loss of sulfur-bound intercalator), were detected in this LC/MS study. The free 2′-deoxynucleosides were the only detectable nonacridine-modified species in the HPLC traces monitored at 254 nm.

Discussion

Reactivity. Conjugate **2** was developed as a therapeutically useful platinum−acridine agent that does not induce cross-links in duplex DNA.[14,15] The design rationale was that the sequence- and groove-specificity of covalent adduct formation with the biopolymer would be dominated by the acridine-based intercalator. This would ultimately enhance the covalent modification of DNA sites normally inaccessible to nontargeted platinum agents. Model reactions between **2** and the potential target nucleoside, dGuo, proceed considerably slower than reactions with double-stranded DNA,[15] suggesting slow hydrolysis of the complex. Intercalative preassociation of acridine in **2** with the duplex, supposedly a rapid process, might accelerate platinum binding by positioning the metal center close to DNA nucleophiles and favor substitution of chloride *without* prior hydrolysis of the Pt−Cl bond. A direct substitution pathway has previously been proposed to account for the binding kinetics of structurally related acridine−cisplatin conjugates.[11b] Notably, the (unwanted) substitution of acridine by dGuo in approximately 15% of the adducts is not observed in reactions of **2** with calf thymus DNA. In native DNA, nucleobase nitrogen apparently replaces chloride with high selectivity, based on the *complete* absence of acridine-free platinum−nucleoside adducts in the enzymatic digests. While Pt−S bonds in adducts containing thioethers have been demonstrated to be unstable in reactions with DNA nucleo-

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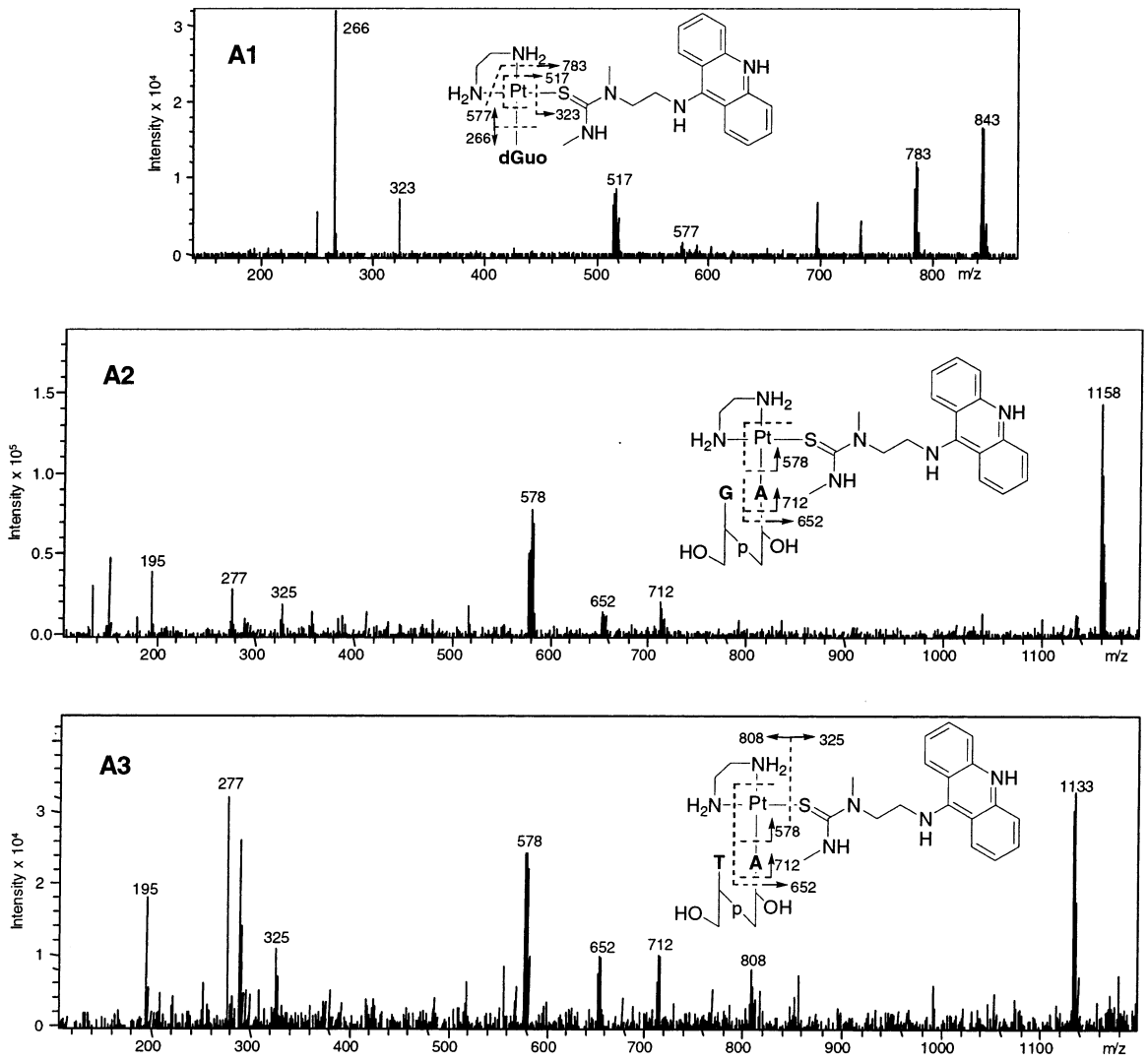

Figure 5. Negative-ion electrospray mass spectrum of adduct **A1** and positive-ion electrospray mass spectra of adducts **A2** and **A3** showing peaks for the adducts [**A1**-4H]$^-$, [**A2**-H]$^+$ and [**A3**-H]$^+$, respectively, and fragment ions resulting from in-source collisionally activated dissociation (CAD). The insets illustrate the observed fragmentation patterns.

philes,[28] the displacement of thiourea sulfur from platinum in **2** by dGuo-N7 is unusual, and only the reverse reaction has been described in the literature.[29]

Modifications of Double-Stranded DNA. In 80% of the adducts formed in calf thymus DNA, platinum in **2** covalently modifies guanine nucleobase. The fact that these adducts are formed in the major groove of DNA suggests that platinum dominates the DNA interactions of conjugate **2** in guanine-specific reactions. The intercalator itself has a high affinity to alternating GC sequences.[17] Although intercalative preassociation of **2** most likely occurs from the minor groove, fast dissociation ("on/off") rates of the conjugate and relatively slow covalent binding might explain why a major portion of the adducts are formed at the high-affinity site, guanine-N7, in the major groove. As stated above, no evidence was found that would indicate transformation of these monofunctional adducts into bifunctional cross-links due to loss of acridine-thiourea. ACRAMTU (**1**) acts as a nonleaving group in reactions with DNA. In contrast to reactions with dGuo, nucleophilic donors such as purine-N7 in a conformationally restricted duplex may not be suitably positioned to displace acridine. Another explanation for the absence of cross-links may lie in the sequence specificity of **1**, which has been demonstrated to intercalate into the guanine-containing 5′-CG/CG and 5′-GA/TC steps in model duplexes. Insertion into the reverse sequences, 5′-GC/GC and 5′-AG/CT, and into the GG/CC base step was not observed.[17] The latter three sequences are the preferred sites of inter- and intrastrand cross-link formation of cisplatin and its analogues.[3,4] Thus, in addition to the substitution-inertness of the Pt−S bond, the intercalation properties of acridine in **2** may contribute to the inability of the metal to form bis(purine-N7) adducts in cisplatin-specific sequences. The complete absence of bifunctional adducts in the enzymatic digests also demonstrates that the chemistry observed in the dGuo model system cannot be extrapolated to double-stranded DNA.

The most intriguing feature of **2** proves to be its ability to induce a high percentage of covalent adenine adducts as

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evidenced by the presence of the platinum-modified GA and TA dinucleotide fragments, **A2** and **A3**. Given the distinct sequence specificity of the acridine derivative **1**, these findings strongly suggest that in ca. 20% of the adducts the intercalator dictates the sites of covalent attachment of platinum in DNA. Consequently, the proposed mechanism of formation of these adducts involves sequence-specific intercalation of the planar chromophore into the GA/TC and TA/TA base steps, respectively, and subsequent platination of adenine. It is proposed, on the basis of the fragmentation behavior in MS/MS experiments, that the modified adenine base in **A2** and **A3** is located at the 3′ terminus. This notion is further supported by the intercalation properties of **1** (NMR).[17] Platination of adenine adjacent to guanine in **A2** is a very unusual binding mode. Guanine-N7 would be expected to be the preferred target site, if adduction occurred in the major groove. Thus, it is proposed that the selective modification of adenine in the GA sequence may occur in the minor groove of DNA in the absence of the competing donor, guanine-N7. In control experiments (Figures S4 and S5) we incubated the single-stranded sequence 5′-GAGAGA-3′ with conjugate **2** and subjected the mixture to enzymatic digestion. Here, the only platinum−acridine-modified DNA fragments detected by HPLC and MS techniques were the mononucleoside adducts, [Pt(en)(ACRAMTU)(dGuo)]$^{3+}$ (major) and [Pt(en)(ACRAMTU)(dAdo)]$^{3+}$ (minor). These results indicate that **A2**, formed in native DNA, is a truly double-strand-specific adduct. The ability of acridine−thiourea to direct platinum into the minor groove of DNA and favor covalent modification of alternative donor sites, such as the N3 position in adenine, is an intriguing possibility. While endocyclic N3 of adenine is a well-established target for various minor groove-directed alkylating agents[30] (including acridine-tethered nitrogen mustards[31]), platinum binding to this site in native B-form DNA is unknown. The only examples of structurally characterized Pt−adenine-N3 binding have been reported for model nucleotides where N1/N7 coordination was prevented by bulky substituents.[32] A recent study[33] of the DNA binding of *trans*-[$PtCl_2$(iminoether)$_2$] using dinucleotide models shows cross-link formation in AG sequences involving adenine-N3. In this case, however, the first binding step involves platination of guanine-N7 followed by closure of the trans chelate with adenine-N3. It is important to note that a critical difference was observed in the fragmentation patterns of **A2** and [Pt(en)(ACRAMTU)(dAdo)]$^{3+}$ in electrospray mass spectra: cleavage of the glycosidic C−N bond is observed for the modified adenine base in **A2** but not in the mononucleotide adduct (see Supporting Information). This observation is consistent with platination of N7 in [Pt(en)(ACRAMTU)(dAdo)]$^{3+}$ preventing protonation of this endocyclic nitrogen. Protonation of N7 is a critical event known to facilitate depurination.[34] This is in contrast to the proposed adenine-N3 binding in **A2**, which would allow protonation of N7 and result in facile loss of adenine base.

We have demonstrated that in the model adduct d(GGAGCTCC)$_2$·(**1**)$_2$ the acridine chromophores intercalate into the GA/TC base steps with the thiourea groups residing in the minor groove of the B-type duplex.[15,17] Similarly, rapid and regiospecific intercalation of acridine in **2** from the minor groove may "hijack" platinum away from its natural target (N7) and promote binding to nucleobase sites in duplex DNA that are usually not favored by platinum. The proposed "pseudo-bifunctional" binding mode in **A2** and **A3** would involve metalation of adenine and intercalation of the chromophore at the 5′ face of the modified nucleobase. Interestingly, no platinum−adenosine mononucleoside adducts were detected in the enzymatic digests, which indicates inhibition of endonucleolytic hydrolysis of the phosphodiester linkage at the GA and TA binding sites. In contrast, the guanine adducts were fully degraded to modified mononucleoside (**A1**). One of the enzymes used in this study, DNase I, is a double-strand-specific endonuclease that associates with DNA through the minor groove.[35] The cleavage activity of this enzyme is known to be efficiently stalled by various minor groove binders (DNase I footprinting[36]). Therefore, the absence of cleavage at the GA and TA sites might indicate that **A2** and **A3** are minor groove adducts.

Conclusions

The targeting of adenine sequences resulting in the formation of monofunctional platinum−adenine adducts breaks one of the longest-standing paradigms in the biocoordination chemistry of platinum anticancer complexes—the requirement for guanine-N7 binding. Conjugate **2** produces a unique array of monofunctional guanine (80%) and adenine (20%) adducts, which opens new avenues in the design of DNA-directed platinum anticancer agents that are truly different from drugs currently in clinical use. We have already demonstrated that **2** exhibits activity in cell lines where cisplatin is a less effective treatment.[14] The covalent adducts detected in this study in cell-free media represent potential cytotoxic lesions at the DNA level, which may trigger suicidal cellular events by interfering with DNA replication and transcription. Many DNA-processing enzymes and gene regulatory proteins associate with DNA through the minor groove, and the modulation of their genomic functions by minor-groove adducts of platinum might have profound biological consequences. The targeting of regions in duplex DNA hitherto inaccessible to platinating agents, such as adenine-containing sequences, would render compound **2** also an interesting tool for studying sequence- and groove-specific interactions between DNA and DNA-binding proteins (e.g., TATA box proteins, TBP[37]).

In summary, we have demonstrated for the first time that it is possible to direct platinum away from its natural target by disfavoring cross-link formation and tethering of the metal to an adenine-affinic, minor groove-directed intercalator. In this report we have identified the covalent adducts of the title compound formed with duplex DNA, which is a crucial step toward an understanding of the molecular recognition between a new platinum anticancer agent and DNA. Molecular biology and high-resolution structural studies are underway to further

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elucidate the sequence context of adduct formation and characterize the adducts and their structural impact in double-stranded DNA.

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Supporting Information Available: Table S1 giving ^{1}H NMR chemical shift assignments for **P1** and **P2**; Figures S1−S5 showing ^{1}H NMR spectral data of incubation mixtures of **2** and dGuo, the MS/MS spectrum of the [**P1**-4H]$^-$ ion, the 2-D gs-COSY spectrum of **P1** (including experimental details of 2-D NMR experiments), the HPLC elution profile of the digested alternating GA hexamer treated with **2**, and the negative-ion electrospray mass spectrum of the adduct [Pt(en)-ACRAMTU(dAdo)]$^{3+}$ (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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