A Versatile Approach Towards Regioselective Platinated DNA Sequences

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Abstract: Undesired N^7 platination of 2′-deoxyguanosine residues at predetermined sites in an oligodeoxynucleotide (ODN) sequence is prevented by applying the sterically demanding diphenylcarbamoyl (DPC) as an O^6-protecting group. The presence of a base-labile oxalyl linker between the immobilized 3′-nucleotide and controlled pore glass (CPG) allows cleavage of the protected ODN from the support and leaves DPC protection unaffected. This method provides an ODN with specifically blocked guanine-N^7 sites for platination. In the hexanucleotides prepared in this study, 5′-GGBGGT-3′(for B = T, C and A), a platinum GG adduct is introduced at G4,G5. These site-specific platinated hexamers were isolated in a yield of 65%, and were fully characterized by using reversed-phase HPLC (high performance liquid chromotography), LCMS (liquid chromatography-mass spectrometry), MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry), PAGE (polyacrylamide gel electrophoresis) and Maxam–Gilbert sequencing analysis.

Keywords: cisplatin · liquid chromatography · oligonucleotides · protecting groups · site-specific platination

Introduction

Platinum coordination compounds play an important role in health care as chemotherapeutics in the treatment of testicular and ovarian cancer.[1] It is now widely accepted that the antitumor activity of cisplatin (*cis*-diamminedichloroplatinum(II)) is related to the ability of this drug to form bifunctional lesions with DNA.[2, 3] The various binding modes of cisplatin to DNA and the resulting structural distortions from B DNA are known, and intrastrand 1,2-GG (G = guanine) crosslinks are predominantly formed.[4, 5] Structural and mechanistic aspects of platinum antitumor drugs have been investigated by elucidation of 3D structures of platinum adducts of oligodeoxynucleotides (ODNs) by X-ray diffraction analysis and solution-based NMR techniques.[6–14]

The construction of selectively platinated DNA adducts by the modification of an unprotected ODN with a platinum complex is hampered by the lack of selectivity of the platination reaction. The synthesis of site-specific platinated ODNs has been restricted only to those with a low purine content.[6–14] Consequently, the study of the diversity of DNA–Pt cross-links is rather limited.

Besides this quest for more heterogeneity within the scope of Pt–DNA adducts, there is a growing interest in using platinated ODNs as therapeutics. Site-specific modifications of ODNs by *trans*-platinum compounds offer potential therapeutical strategies, like gene modulation by antisense and antigene methodologies.[15–18]

The interest and relevance of the site-specific metallation of ODNs has recently been reviewed by Grinstaff et al.[19] Regioselective ruthenium- and osmium-modified ODNs have been prepared by means of solid-phase DNA synthesis by Tor et al.,[20] and by Grinstaff et al.[21] from phosphoramidite derivatives of Ru/Os-modified nucleosides. However, this strategy fails, due to the incompatibility of the amidite function with the coordinated platinum complex [22, 23] in the preparation of a N^7-platinated phosphoramidite derivative of 2′-deoxyguanosine. A recent contribution from this laboratory revealed that N^7-platination of 2′-deoxyguanosine can be effectively prevented by the presence of an O^6-diphenylcarbamoyl (DPC) protecting group.[24]

A new and convenient approach to the preparation of site-specific N^7-platinated ODNs is reported, by using the fully protected 2′deoxyguanosine derivative **1a** (see Figure 1) in preventing N^7-platination with platinum complex **2** (see Figure 1).

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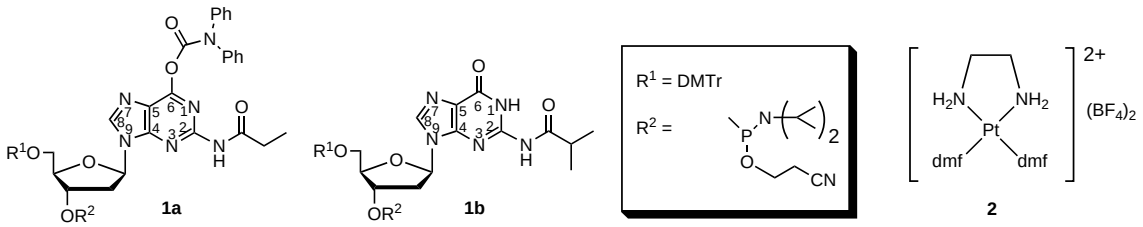
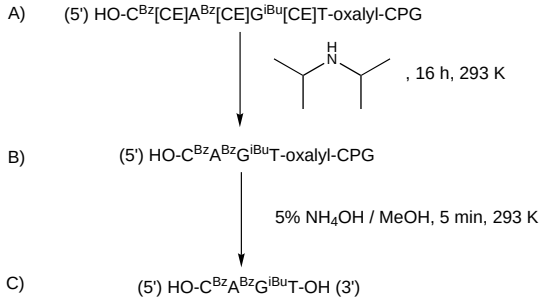


Figure 1. Structures of the fully O^6-DPC-N^2-Pr-protected and partially N^2-*i*Bu-protected dG units **1a** and **1b**, respectively, as well as $[Pt(en)(dmf)_2](BF_4)_2$ (**2**), an analogue for cisplatin (*cis*-$[Pt(NH_3)_2Cl_2]$).

Results and Discussion

As mentioned above,[24] N^7-platination of 2′-deoxyguanosine can be completely prevented by the presence of the sterically demanding diphenylcarbamoyl (DPC) protecting group at the O^6-postion of the guanine base. It is also well known that platination of ODNs occurs most efficiently in aqueous solutions. Unfortunately, a solution-phase synthesis of ODNs containing, both O^6,N^2- and N^2-protected dG units is an intrinsically laborious and rather time-consuming process. On the other hand, a successful stepwise solid-phase synthesis (SPS) of ODNs containing O^6-DPC-N^2-Pr- and N^2-*i*Bu-protected D-guanosine units (i.e., **1a** and **1b**, respectively) has to meet the following two demands. Both the release of the required ODN from the solid support and the unmasking of the cyanoethyl (CE) phosphate protecting groups must be compatible with the presence of the O^6-DPC protecting group. Nearly a decade ago, Letsinger et al.[25] reported that a partially protected ODN, immobilized on controlled pore glass (CPG) by a rather labile oxalyl link, could be converted into an *N*-acyl protected ODN by the two-step process depicted in Scheme 1. For example, treatment of the immobilized ODN fragment **A** with dry diisopropylamine led to the



Scheme 1. Solid-phase synthesis of an *N*-acyl protected ODN, in which [CE] represents the β-cyanoethyl phosphotriester ($3' \rightarrow 5'$) internucleoside link, Bz an *N*-benzoyl and *i*Bu an *N*-isobutyryl group.

exclusive elimination of the β-cyanoethyl (CE) phosphate protecting groups to give the charged immobilized fragment **B**. Subsequent selective cleavage of the oxalyl link, leading to the *N*-acyl protected fragment **C**, could be effected in less than five minutes by treatment of the immobilized sequence **B** with 5% ammonium hydroxide in methanol.

It is evident that the earlier formulated demands for a successful SPS of ODNs, that contain O^6,N^2- and N^2-protected d-G units would only be met if the O^6-DPC group survived the two-step deblocking protocol (i.e., conversion of **A** into **C**) of Letsinger. This last requisite was addressed by subjecting the O^6-DPC-N^2-Pr-protected D-guanosine derivative **1a** ($R^1 = R^2 = H$) to several basic conditions, the results of which are recorded in Table 1.

Table 1. Relative stability of the O^6-DPC-group in O^6-DPC-N^2-Pr-dG (**1a**, $R^1 = R^2 = H$) under basic conditions.

Entry	Conditions	$t_{1/2}$[a][min]
1	diisopropylamine (dry)	> 10000
2	0.1M NaOH in H_2O/dioxane, 1/1, v/v	240
3	conc. NH_4OH/solvent [b], 1/1, v/v	90

[a] Estimated by TLC analysis. [b] MeOH, *i*-propanol or dioxane.

Firstly, it was gratifying to establish that the O^6-DPC group was fully compatible (see entry 1) with the unmasking of the CE-protecting groups. Also, the DPC group will survive the cleavage of the oxalyl linker by using either sodium hydroxide (0.1M) in methanol for 1 minute (see entry 2), or ammonium hydroxide (5%) in methanol for 5 minutes (see entry 3). Moreover, complete removal of the DPC and *N*-acyl protecting groups in the final stage of the synthesis can be performed most efficiently with concentrated ammonium hydroxide.

At this stage, having established the compatibility of the O^6-DPC protecting group with the Letsinger's two-step deblocking process, the SPS of the ODN fragment **5a** ($B = C^{Bz}$) and its conversion into the monoplatinated target compound **7a** ($B = C$) were first investigated (see Scheme 2). The construction of the partially protected ODN fragment **5a** ($B = C^{Bz}$) commences by immobilization of 5′-*O*-dimethoxytritylthymidine through the labile oxalyl linker to controlled pore glass (CPG) to give compound **3**.[25] By using an automated DNA synthesizer, sequential elongation of compound **3** with commercially available phosphoramidite building blocks and the known O^6-DPC,N^2-Pr-protected D-guanosine derivative **1a**,[26] provided the fully immobilized fragment **4a** ($B = C^{Bz}$). The overall coupling efficiency was found to be ~90%, as gauged spectrophotometrically by the released DMT cation.

Treatment of immobilized **4a** ($B = C^{Bz}$) with dry diisopropylamine for 16 h at 293 K was followed, after extensive washing of the resin with acetonitrile, by a short treatment with sodium hydroxide (0.1M). Neutralization of the reaction after 1 minute at 293 K, with Sephadex (H^+-form), gave, as evidenced by LCMS analysis, the partially protected ODN **5a** ($B = C^{Bz}$) as the major product (see Figure 2, trace A). Platination of crude **5a** ($B = C^{Bz}$) with the bifunctional platinating agent **2** proceeded smoothly (see Figure 2,

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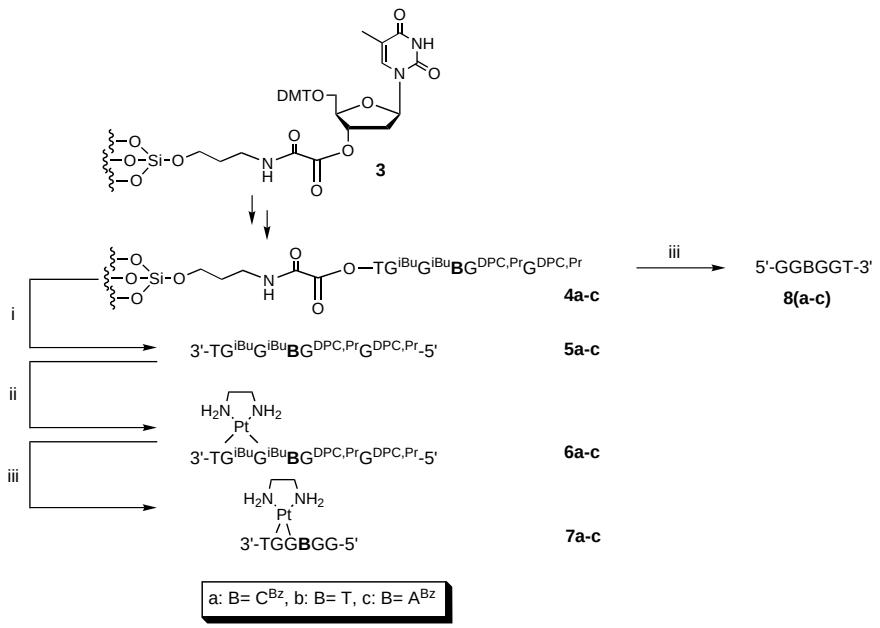

Scheme 2. Synthetic pathway to compounds **4**–**8**. i) a) Dry diisopropylamine, 16 h, RT; b) 0.1 M NaOH, H_2O/1,4 dioxane, 1/1, v/v, 1 min; Sephadex H^+. ii) 100–300 μM $[Pt(en)(dmf)_2](BF_4)_2$, in DMF or H_2O, 16 h, RT. iii) Conc. NH_4OH, 16 h, 50 °C.

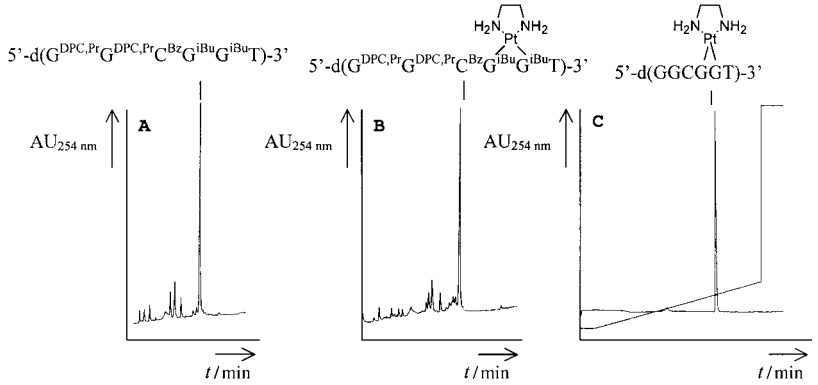

Figure 2. Reversed phase HPLC analysis of the crude hexamers 5′-d(GGCGGT)-3′ A) **5a**, and B) **6a**, and C) the purified **7a**.

trace B) to produce the crude monoplatinated adduct **6a** (B = C^{Bz}). Ammonolysis of the latter adduct gave, after anion-exchange and reversed-phase HPLC, the homogenous target compound **7a** (B = C) in an overall yield of 14% (see Figure 2, trace C). The homogeneity of **7a** was also confirmed (see Figure 3C) by PAGE.

The presence of a platinum lesion in **7a** (B = C) was indirectly established by the following well-known deplatination procedure.[23] Hence, treatment of **7a** (B = C) with sodium cyanide (0.3 M) at pH 9 for 16 h at 37 °C, gave the fully deplatinated hexameric ODN, which co-migrated (in anion-exchange HPLC and PAGE) as expected with the ODN fragment **8a** (B = C, see Scheme 2) obtained after ammonolysis of the immobilized hexamer **4a** (B = C^{Bz}).

In addition, MALDI-TOF mass spectrometry analysis (see Experimental Section) of **7a** (B = C) revealed the presence of one ligated {Pt(en)} moiety. More importantly, the presence of the pCp[GpGPt(en)]pT fragment (as $[M^{2+}+2H]$) in the ESMS spectrum of **7a** (B = C) is a clear indication of a {Pt(en)} cross-link between G4 and G5. Moreover, regioselective platination at G4,G5 was also unambiguously ascertained by Maxam-Gilbert sequencing analysis which showed (Figure 3 D) absence of platination at G1,G2.

The efficacy of the methodology is also well illustrated by the synthesis of the monoplatinated hexamers **7b** (B = T) and **7c** (B = A), which were both isolated as homogeneous compounds as gauged by reversed-phase HPLC analysis (see Figure 3 panels A and B), PAGE (panel C) and Maxam–Gilbert sequencing (panel D).

Conclusion

These results show for the first time, a successful approach to the synthesis of regioselective platinated DNA sequences. This novel method is reproducible and allows platination of heterogeneous, that is, multiple A and G-containing sequences.

It is anticipated that other site-specific platinated oligonucleotides, such as *trans*-Pt-1,3-GXG adducts for potential antisense applications, as well as other ligands, may be constructed in a similar manner.

Experimental Section

General: Compound $[Pt(en)Cl_2]$ was synthesized according to literature procedures and converted into the reactive species $[Pt(en)(dmf)_2](BF_4)_2$ by treatment with a 1.95 equivalent of $AgBF_4$ in DMF.[28, 29] The fully protected phosphoramidite building block **1a** was prepared according to an earlier described procedure.[26, 27] Oxalyl-CPG was prepared as described and was used, loaded with DMTdT, as the solid support.[25] The resin loading was determined by treating the resin (2.24 mg) with trichloroacetic acid (3%) in dichloromethane, and by measuring the concentration of the released trityl cation (λ_{max} 505 nm). The resin loading was 51 μmol g^{-1}.

All chemicals, apart from the D-nucleotide building units, were purchased from Aldrich Chemicals. TLC analysis was performed on Merck Silica gel 60 F_{254} visualized at 254 nm. Analytical anion-exchange HPLC was performed on a Mono Q HR 5/5 column (Pharmacia), elution at pH 12.0: step gradient of buffer A (0.01 M NaOH) to buffer B (0.01 M NaOH + 1.2 M NaCl). For analytical anion-exchange HPLC analysis of DPC protected species, an alternative buffer system was applied; 25 mM NaOAc in CH_3CN:H_2O (2/8) (A) → 1 M NaCl in 25 mM NaOAc/CH_3CN (8/2) (B).

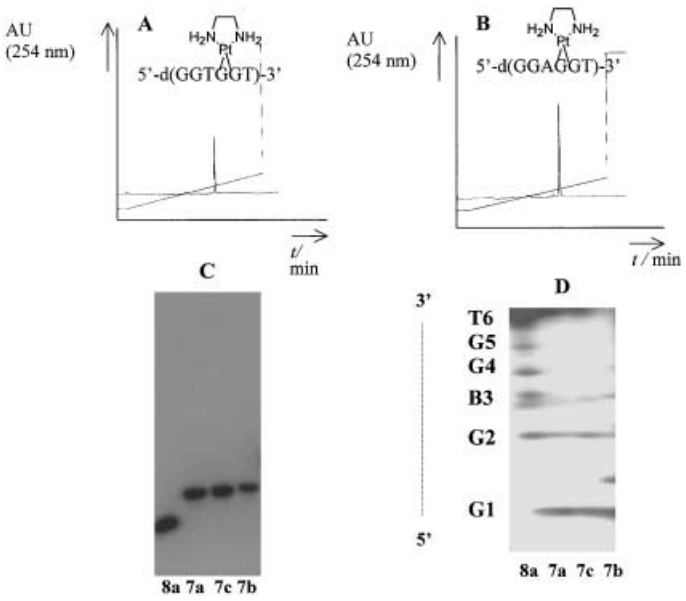

Figure 3. Reversed-phase HPLC analysis of A) **7b** (B = T) and B) **7c** (B = A). C) PAGE (20%) of platinated hexamers, **7a-c** and a nonplatinated control **8a** (deprotected derivative thereof, i.e., d(5′-GGCGGT-3′)) and D) Maxam – Gilbert sequencing analysis proving platination at G4 and G5 (absence of platination at G1 and G2 is evident).

Analytical reversed phase chromatography on purified platinated hexamers, (compounds **7a**,**b** and **c**). Column: Alltima C18, 5 μM, 4.6 × 150 mm. Buffers A: 50 mM TEAA pH 7.0 and B: 50 mM TEAA pH 7.0/CH_3CN (1/3 v/v). Flow rate of 1.0 mL min^{-1} at 40 °C.

LCMS was performed by using a JASCO LCMS system coupled to an ABI 165 SciEx ES-MS operating in positive mode. Reversed-phase column: Alltima C18, 5 μm, 4.6 × 150 mm. Buffers A: 10 mM NH_4Oac; B: 20% CH_3CN in 10 mM NH_4OAc.

Synthesis of oligodeoxynucleotides: The polymer-supported synthesis of DPC-protected G-containing DNA fragments (see Scheme 1) was performed on a fully automated synthesizer (Pharmacia Gene Assembler Special), with phosphoramidite **1a** and commercially available (Perseptive Biosystems, USA) 2′-deoxynucleoside 3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidites. Oxalyl-controlled pore glass (CPG-AP), loaded with DMTdT, was used as the solid support. Deprotection of the cyanoethyl protecting groups was established by diisopropylamine for 16 h at RT. Cleavage of the base-protected oligodeoxynucleotide from the solid phase was performed by treatment of the CPG with 0.1 M NaOH in H_2O/1,4-dioxane, 1/1, v/v (1 min, syringe; quantitative cleavage with 0.01 M NaOH required 10 min) and immediate quenching with Sephadex H^+, a weakly acidic ion-exchanger. The solid support was rinsed with H_2O/CH_3CN (8/2, v/v). The solution was concentrated and a stock solution of the ODN was prepared in H_2O/MeOH (8/2, v/v). ODN sequences were synthesized starting on a 1 μmol scale. A typical preparation, that is, the synthesis of **4a**(B = C^{Bz}), yielded 21.9 mg of **4a** (329 nmol, 32.9% yield). Subsequent deprotection by ammonolysis, ion exchange chromatography, and reversed phase chromatography purification, yielded compound **8a** (B = C) in 21% yield.

Platination: Platination of the base-protected ODN (100 – 300 μM) was effected by treatment of the stock solution with 1.0 equiv of $[Pt(en)(OH_2)_2](BF_4)_2$ for 16 h at RT. The reaction was followed by ion exchange, as well as reversed phase HPLC. The crude mixture was concentrated under reduced pressure (8 mbar, 4 h, 30 °C) and complete deprotection of the platinated ODN was effected with concentrated ammonia in a sealed vial and heated for 16 h at 50 °C. Purification was performed by fast protein liquid chromatography (FPLC) with a Pharmacia (Uppsala, Sweden) LCC-500 liquid chromatograph. The appropriate fractions were collected and neutralized with acetic acid. Desalting and further purification was effected by reversed phase chromatography (Lichrospher C18, Merck, Germany). In a typical experiment, that is, the preparation of **7a** (B = C), 1.23 mL of a 220 μM solution of **4a** (271 nmol) in H_2O/MeOH (8/2 v/v mixture) was reacted with 543 μL of a 500 μM solution of $[Pt(en)(OH_2)_2](BF_4)_2$ (271 nmol), a 200-fold H_2O-diluted solution from the 0.1 M DMF stock. The final purified product, **7a** (B = C), was isolated in 14% yield.

Purity of the target compounds **7a – c** was determined by analytical reversed-phase HPLC, LCMS, and 20% polyacrylamide gel electrophoresis. (MALDI TOF) MS analysis was performed at Eurogentec (Belgium).

Hexamer 7a: 5′-GG**C**[GGPt(en)]T-3′
LCMS: 1052 [M^{2+}+2H], 1063 [M^{2+}+H+Na], 1074 [M^{2+}+2Na], 702 [M^{3+}+3H], 710 [M^{3+}+2H+Na]. Fragmentation: GpGpC[pGpGPt(en)]pT → GpG + pCp[GpGPt(en)]pT. The latter fragment was visible as [M^{2+}+2H] 763 and its monosodium species [M^{2+}+H+Na] 774. MALDI-TOF, positive mode, found: 2100 [M^++H]; calcd: 2103. Retention time analytical reversed phase HPLC: 11.5 min.

Hexamer 7b: 5′-GG**T**[GGPt(en)]T-3′
LCMS: 1058 [M^{2+}+2H], 1069 [M^{2+}+H+Na], 1080 [M^{2+}+2Na], 706 [M^{3+}+3H], 714 [M^{3+}+2H+Na], 721 [M^{3+}+H+2Na], 728 [M^{3+}+3Na], 736 [M^{3+}+-H+4Na]. Fragmentation: GpGpTp[GpGPt(en)]pT → GpG + pTp[GpGPt(en)]pT. The latter fragment was visible as [M^{2+}+2H] 769 and its mono-sodiated species [M^{2+}+H+Na] 780. MALDI-TOF, positive mode, found: 2116 [M^++H]; calcd: 2118. Retention time analytical reversed Phase HPLC: 10.4 min.

Hexamer 7c: 5′-GG**A**[GGPt(en)]T-3′
LCMS : 1064 [M^{2+}+2H], 1075 [M^{2+}+H+Na], 1086 [M^{2+}+2Na], 710 [M^{+3}+H], 718 [M^{3+}+2H+Na], 725 [M^{3+}+H+2Na]. Fragmentation: GpGpA[pGpGPt(en)]pT → GpG + pAp[GpGPt(en)]pT. The latter fragment was visible as [M^{2+}+2H] 774 and its mono-sodiated species [M^{2+}+H+Na] 785. MALDI-TOF, positive mode, found: 2119 [M^++H]; calcd: 2126. Retention time analytical reversed phase HPLC: 11.7 min.

Poly acrylamide gel electrophoresis and Maxam – Gilbert sequencing: Hexamers were labeled 5′ in a kinase reaction with γ-^{32}P-ATP (Amersham-Pharmacia Biotech 6000 Ci mmol^{-1}). Labeled hexamers were run on a 20% (19:1) 7 M urea polyacrylamide gel (8 × 10 cm) in 1 × TBE buffer. The chemical degradation of guanine of the platinated hexamers was carried out according to Maxam & Gilbert, with a modification for the time to 15 min at 37 °C.[30] Samples were run on a 20% (19:1) 7 M urea polyacrylamide gel (30 × 40 cm) in TBE buffer for 2400 V × H at 35 mA.

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