Monofunctionally *trans*-Diammine Platinum(II)-Modified Peptide Nucleic Acid Oligomers: A New Generation of Potential Antisense Drugs**

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We dedicate this work to the memory of our friend and colleague Marc Leng.

Abstract: A solid-phase approach is described that provides facile access to monofunctionally *trans*-Pt^{II}-modified PNA oligomers of arbitrary sequence for potential use both in antigene and antisense strategies. The approach includes the synthesis of a platinated building block **1** and its subsequent incorporation into three different PNA oligomers **5**–**7** by solid-phase synthesis. In a model cross-linking reaction one of the latter is found to recognize sequence-specifically a target oligonucleotide **8** and to cross-link to it. The resulting structure is the *trans*-Pt^{II}-cross-linked PNA/DNA duplex **9** as revealed by mass spectrometry in combination with a Maxam-Gilbert sequencing experiment.

Keywords: antisense agents · nucleic acids · peptide nucleic acids · platinum · solid-phase synthesis

Introduction

Peptide nucleic acid (PNA), a DNA mimic in which the sugar–phosphate backbone has been replaced by *N*-(2-aminoethyl)glycine units,[1] is a very promising candidate for the regulation of gene expression (e.g. antigene and antisense strategies).[2, 3] This property is based on its strong and specific binding to target DNA or RNA sequences[4] in combination with high chemical and biological stability.[5] The potential application of PNA in antisense strategies is based on steric blockage of ribosomes or essential translation factors. RNase H activation is, in contrast to other antisense oligonucleotide (analogues), not involved.[6, 7] While triplex-forming homopyrimidine bis-PNAs cause sufficient steric blockage when targeted to the coding region of mRNA,[6, 7] duplex-forming mixed pu/pym PNAs cause antisense effects mainly due to sequences around the 5′-untranslated region of RNA.[7–10] In the latter case, steric blocking of sequences within the coding region was found to be poor due to insufficient stability of PNA–DNA duplexes against the moving ribosome.[7–10] Thus, targeting mixed pu/pym sequences within the coding region of mRNA, which is highly relevant for an efficient application in antisense strategies, is not easily accomplished.

To increase the affinity of an antisense/antigene oligonucleotide (analogue) to its target, the principle of cross-linking the two strands is the method of choice.[11–14] Amongst the different cross-linking agents, metal complexes of suitable geometry and coordination behaviour seem especially promising.[14] For example, recent studies have shown quite impressively that a linear *trans*-a_2Pt^{II} unit ($a = NH_3$) unit is able to cross-link two oligonucleotide strands sequence-specifically in a thermodynamically stable *and* kinetically inert mode without large steric distortion.[15–19] The resulting *trans*-Pt^{II} cross-linked oligonucleotide/target duplexes exhibit enhanced thermal stability[16, 18] with increased steric blocking of the target sequence. The impact of this principle in an antisense strategy was recently convincingly illustrated. It was

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[**] Abbreviations: PNA, peptide nucleic acid; pu, purine base; pym, pyrimidine base; *trans*-Pt^{II}, *trans*-a_2Pt^{II} with $a = NH_3$ or amine; PEG-PS, polyethylene glycol-derivatized polystyrene; Fmoc, 9-fluorenylmethyloxycarbonyl; Bhoc, benzhydryloxycarbonyl; HATU, 2-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; D*i*PEA, *N*,*N*-diisopropylethylamine; TFA, trifluoroacetic acid; DMS, dimethylsulfate; Boc, *tert*-butyloxycarbonyl; NMP, *N*-methyl-2-pyrollidone; DTT, dithiothreitol

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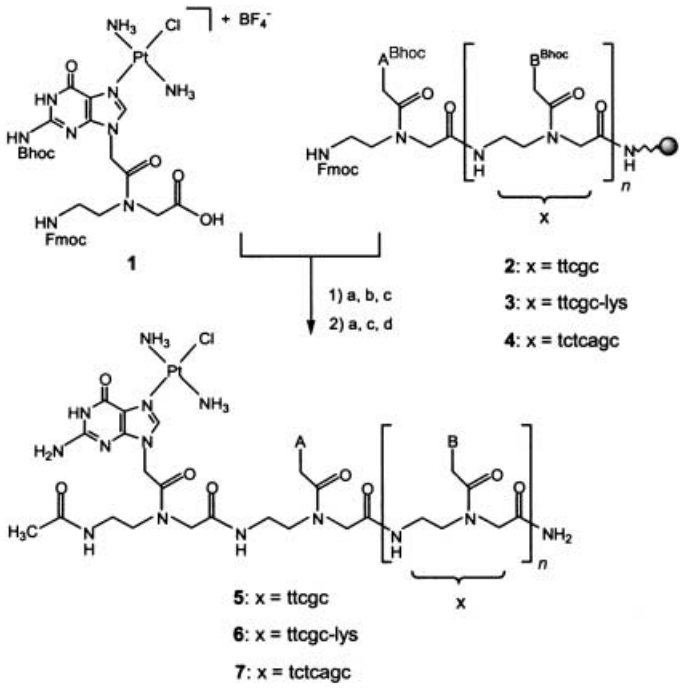
found that 2′*O*-methyloligoribonucleotides, cross-linked via *trans*-a_2Pt^{II} units to a target sequence within the coding region of mRNA, caused an effective arrest of translation both in vitro and in cells.[10, 20, 21] A major limiting factor for the successful application of this principle for in vivo gene regulation is the lack of an efficient synthesis of site-specifically *trans*-Pt^{II}-modified deoxyoligonucleotide (analogues). Studies directed to this aim[14, 22] have been pursued and significant progress has already been achieved by incorporation of preplatinated building blocks into oligonucleotides by solid-phase DNA-synthesis methodologies.[23–25] However, a really efficient method is not yet available.

Here, we report a solid-phase approach that, in a novel way, provides facile access to monofunctionally *trans*-Pt^{II}-modified peptide nucleic acid oligomers of *any* desired sequence with cross-linking ability. Combination of the promising antisense properties of PNA with the favorable cross-linking properties of transplatin yields novel platinated antisense drugs that were specifically designed to block sequences even in the coding region of mRNA.

Results

Synthesis and characterization of the monofunctionally *trans*-Pt^{II}-modified PNA oligomers: First, the *trans*-Pt^{II}-modified building unit **1** (Scheme 1) was synthesized in high yield (75%) by the reaction of *trans*-$[Pt(NH_3)_2Cl(DMF)]BF_4$ with 1 equiv. Fmoc/Bhoc-PNA G. The identity of **1** was firmly established by ^1H, ^{195}Pt NMR, $^1H,^1H$ NOESY and $^1H,^1H$ COSY spectroscopy as well as LC–MS. Next, the compatibility of **1** with a well-established, automated PNA synthesis protocol[26] was confirmed by subjecting **1** to the various reactants of this protocol and probing potential changes in the Pt^{II} coordination sphere by ^{195}Pt NMR spectroscopy.



Scheme 1. Solid-phase synthesis of monofunctionally *trans*-Pt^{II}-modified PNA oligomers; a) piperidine, b) HATU/D*i*PEA, c) Ac_2O/D*i*PEA, d) TFA/*m*-cresol.

At this stage, sequential elongation of Rinkamide, anchored to functionalized PEG-PS resin with Fmoc/*N*-Bhoc-protected building units by using the coupling reagent HATU, and subsequent reaction of the resulting resin-bound fully protected PNA oligomers **2**, **3** and **4** with excess **1** in the presence of *Di*PEA gave immobilized **5, 6** and **7**, respectively. Removal of the *N*-Bhoc protecting groups from the nucleobases and concomitant release from the solid support was effected with TFA/*m*-cresol (4:1). Crude monofunctionally *trans*-Pt^{II}-modified PNA oligomers **5, 6** and **7** were obtained in good yields of 50% each as gauged by LC–MS. The major side products were, in all cases, the respective unreacted ($n-1$)-mers; this indicates that coupling of **1** to the immobilized PNA oligomers **2**, **3** and **4** did not proceed to completion. Isolation of the pure conjugates **5**, **6** and **7** was subsequently accomplished by reversed-phase HPLC.

Cross-linking reaction with a complementary oligonucleotide: An prerequisite for a potential application of the synthesized monofunctionally *trans*-Pt^{II}-modified PNA oligomers **5**, **6** and **7** in antisense strategy is their sequence-specific cross-linking reaction with a complementary RNA target. In a model experiment, the cross-linking of *trans*-$[(NH_3)_2Pt(g\text{-}N7\text{-}attcgc)Cl]^+$ (**5**) with the deoxyoligonucleotide target 5′d(GCGAATG) (**8**) was investigated. Instead of RNA, DNA was used in order to avoid problems with RNA hydrolysis. Furthermore, the deoxyoligonucleotide **8** is not entirely complementary to **5** in that it contains a 3′-terminal guanine instead of a cytosine. This modification was chosen on purpose to exploit the greater affinity of Pt^{II} for guanine compared to cytosine[18, 27] and to increase the rate of interstrand cross-link formation between **5** and **8**. PNA, which contains all four natural nucleobases, hybridizes to complementary oligonucleotides according to the Watson–Crick base pairing rules.[28] Therefore, efficient hybridization of **5** with **8** should lead to the conjugate **9** with an antiparallel orientation of the strands. In the reaction of *trans*-$[(NH_3)_2Pt(g\text{-}N7\text{-}attcgc)Cl]^+$ (**5**) with 5′d(GCGAATG) (**8**) at pH 6.5 in a $NaClO_4$/DMF (0.1M, 5:1, *v*/*v*) at RT, the formation of a single new product was observed by HPLC analysis. It was subsequently isolated by HPLC and analyzed by MALDI-TOF MS (Figure 1). Here a peak at $m/z = 4325.07$ was observed that can be assigned to a singly positively charged *trans*-Pt^{II} cross-linked PNA/DNA hybrid. Additionally, peaks

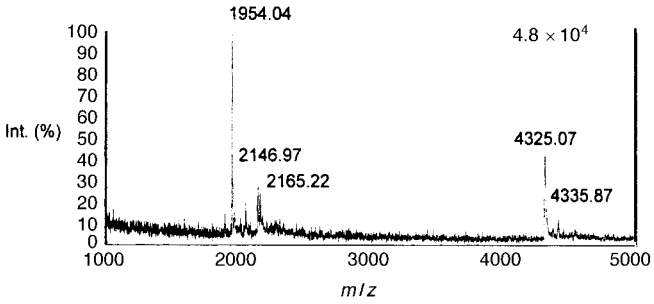


Figure 1. MALDI-TOF spectrum of **9**

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for the unplatinated gattcgc (**5**) and the unplatinated 5′d(GCGAATG) (**8**) were detected, probably due to fragmentation of **9** during the MALDI-TOF experiment. Further isolated HPLC fractions contain unreacted 5′d(GCGAATG) (**8**) and a product with $m/z = 2178.6$, which can be assigned either to **5** with the chloro ligand being removed during the MALDI-TOF experiment (fragmentation) or as a product resulting from a suicide reaction (formation of an intrastrand cross-link within **5**).

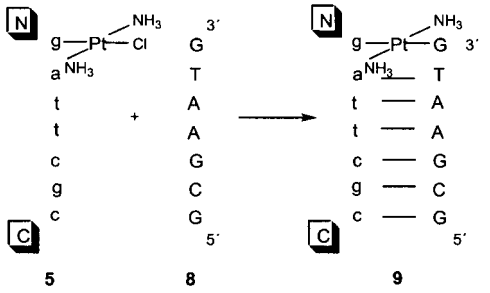
Identification of the cross-linked residue within the DNA strand of 9 by chemical mapping: The identity of the platinated residue within the DNA strand was revealed by comparison of the DMS sequencing patterns of compounds **8** and **9**. The N7 atoms of guanines react with DMS, which sensitizes the corresponding deoxyribose moieties to cleavage by hot piperidine (Maxam and Gilbert sequencing[29]). However, DMS is not expected to react with platinated G residues, their N7 atoms being no longer accessible.[29, 30] As platinated guanines are not cleaved by hot piperidine,[30] their presence is revealed by gaps at corresponding positions in the sequencing pattern. Such a gap is evident in the DMS sequencing pattern of compound **9** (compare lanes 2 and 5 in Figure 2) and

Figure 2. Autoradiogram of a 24% polyacrylamide/7M urea gel of the products of the reaction between DMS or Fe–EDTA and compound **8** or **9**. The 5′ ends of the DNA fragments were labeled with ^{32}P. Lanes: 1 and 6: unmodified compound **8**; 2 and 5: fragments generated by the reaction with DMS followed by treatment with piperidine; 3 and 4: fragments generated by the reaction with hydroxyl radicals produced by Fe–EDTA and hydrogen peroxide. In this case, the cleavage reaction generates two fragments of slightly different electrophoretic mobilities for every position (4). The base sequence of the DNA strand is shown between the two panels. Note that the smaller fragments were run out of the gel in order to better resolve the other fragments. The star refers to the cross-linked G residue.

corresponds to the 3′-end G residue of the DNA strand. The position of the platinated residue was confirmed by Fe–EDTA mapping. The hydroxyl radicals generated by the reaction of the EDTA complex of iron(II) with hydrogen peroxide cleave the DNA phosphodiester backbone at the level of the deoxyriboses.[31] Because the base moiety is released during the Fe–EDTA reaction,[31] the location of the platinated residue is usually revealed by the absence of signal at the next position.[32] As shown in Figure 2, there is no such a gap in the Fe–EDTA sequencing pattern of compound **9** up to the residue of the DNA strand closest to the 3′ end. This result confirms that the 3′-guanine of the DNA strand is the cross-linked residue. Taken together, these results clearly demonstrate that the isolated product is the interstrand cross-linking product **9**.

Discussion

Our results show that the newly synthesized platinated building block **1** is fully compatible with the employed PNA synthesis protocol[26] with regard to ligand-exchange reactions. Thus, conjugation of **1** to the N terminus of the immobilized PNA oligomers **2**, **3** and **4** leads to the synthesis of the monofunctionally *trans*-Pt^{II}-modified mixed pyrimidine/purine PNA oligomers **5**, **6** and **7**. The potential use of these cross-linking reagents in antisense strategy has been demonstrated by a model cross-linking reaction of the monofunctionally *trans*-Pt^{II}-modified PNA oligomer *trans*-[$(NH_3)_2$Pt(g-*N7*-attcgc)Cl]$^+$ (**5**) with the complementary oligonucleotide 5′d(GCGAATG) (**8**). Here sequence-specific recognition has been seen to take place *prior* to formation of an interstrand cross-link leading to formation of the cross-linked PNA/DNA duplex **9** (Scheme 2). In the case of insufficient or nonspecific



Scheme 2. Cross-linking reaction of **5** with **8**.

recognition between **5** and **8**, several cross-linking products with platination sites different from the 3′G of the DNA part should have formed, which in fact were not observed by HPLC and gel electrophoresis. Although, according to HPLC analysis, the cross-linking reaction proceeded slowly and in low yields (ca. 5%) under the reaction conditions, it became clear that i) only a single product was detected and that ii) this product was the expected interstrand cross-linking product as confirmed by MALDI-TOF MS and the Maxam–Gilbert sequencing experiment. The slowness of the cross-linking reaction is, at least in part, due to the low concentrations applied, which in turn were a consequence of the poor solubility of the platinated PNAs.

It is also conceivable that hindered rotation about the G-N(9)–C(9) bond in **5** is responsible for the low yield of **9**. Formation of an interstrand cross-link requires rotation of this bond within **5** to place the *trans*-a$_2$Pt^{II}Cl unit in proximity of

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the N7 position to the 3′G of **8**. Indeed, at least for building block **1**, hindered rotation about the G-N(9)–C(9) has been observed by temperature-dependent ^{1}H NMR studies. For example, the ^{1}H NMR spectrum (data not shown) of building block **1** in [D_7]DMF exhibits a doubling of resonances for the G-H8 and G-N(9) methylene protons. Coalescence of the G-H(8) resonance occurs even at 338 K and does not coincide with the coalescence of the G-N(9) methylene proton resonances, which occurs at significantly higher temperature (>360 K). This suggests that below 338 K the nucleobase rotation about the G-N(9)–C(9) bond is slow. It is assumed that hindered rotation about the G-N(9)–C(9) bond in **5** prevents formation of the interstrand cross-link. Indeed cross-linking of longer *trans*-Pt^{II}-modified PNA sequences at higher temperatures or the use of a more flexible PNA backbone[33] may circumvent the problem of hindered rotation.

In summary, the methodology described above allows for the first time a facile preparation of monofunctionally *trans*-Pt^{II}-modified mixed pu/pym PNA oligomers of arbitrary sequences in rather good yields. These have been shown to cross-link sequence-specifically with a target oligonucleotide. Although the platinated PNAs synthesized are not ideal yet as far as possible medical applications are concerned (poor solubility, requirement of DMF), our approach allows for the first time the synthesis of PNA-based antisense drugs with cross-linking ability. It thus opens up the way to the future design of a new class of useful chemical peptide nucleic acid probes[34] and antisense reagents.

Experimental Section

Materials: K_2PtCl_4 was purchased from Heraeus. *trans*-Pt$(NH_3)_2Cl_2$ was synthesized according to a literature procedure.[35] Fmoc/N-Bhoc-protected PNA monomers were obtained from PE Biosystems; Fmoc/Boc-protected L-lysine was from NovaBiochem; TFA and *m*-cresol were from Fluka. Solid-phase PNA synthesis was performed on a 433A Peptide Synthesizer (Applied Biosystems) with PEG-PS resin functionalized with a RINK linker as the solid support, or on a Pharmacia Gene Assembler by using highly cross-linked polystyrene beads as the solid-support.

Synthesis of building block 1: *trans*-[Pt$(NH_3)_2$Cl(PNA-G)]BF_4 (**1**) was synthesized in 75% yield by treating *trans*-[Pt$(NH_3)_2$Cl(DMF)]BF_4, obtained in situ from *trans*-[Pt$(NH_3)_2Cl_2$] and $AgBF_4$, with 1 equiv. Fmoc/Bhoc-PNA G in DMF. ^{195}Pt NMR ([D_7]DMF): $\delta = -2289$; the complete assignment of the protons was corroborated by a ^{1}H,^{1}H COSY experiment; ESI-MS: m/z: 1006 [M^+].

Solid-phase PNA synthesis: Assembly of the PNA sequences **2**–**4** and the *trans*-Pt^{II}-modified PNA oligomers **5**–**7** was carried out on a 433 A Peptide Synthesizer (Applied Biosystems) according to a slightly modified literature procedure.[26] PEG-PS resin functionalized with a RINK linker (loading 0.2 mmol g^{-1}) was used on a 1 μmol scale as the solid support. Assembly of the monofunctionally *trans*-Pt^{II}-modified PNA oligomers **5**, **6** and **7** was performed by using solutions of Fmoc/N-Bhoc-protected (0.1M) PNA monomers (and Fmoc/Boc-protected L-lysine in the case of **6**) and *trans*-[Pt$(NH_3)_2$(Fmoc/N-Bhoc-G)Cl]$^+BF_4^-$ (**1**) in NMP. Prior to the coupling, the monomers were preactivated by mixing equal amounts of the PNA monomer (25 equiv. per μmol support), HATU and D*i*PEA solutions for 1 min. The protocol for one PNA chain extension cycle comprised 1) Fmoc deprotection: 20% piperidine in NMP (1 mL, 1 × 1 min, 4 × 0.5 min), 2) wash: NMP (5 × 1.5 mL), 3) coupling: PNA + HATU + D*i*PEA in NMP, 15 min; 4) wash: NMP (5 × 0.5 mL), 5) capping: Ac_2O (0.5M)/D*i*PEA (0.125M) in NMP and 6) wash: NMP (5 × 0.5 mL). At the end, a Fmoc protection was carried out (20% piperidine in NMP (1 mL, 1 × 1 min, 4 × 0.5 min)) followed by a final wash (NMP (5 × 1.5 mL), CH_2Cl_2 (5 × 2 mL)). Deprotection and cleavage from the solid support were effected by treatment with TFA/*m*-cresol (4:1 *v*/*v*) for 1 h.

Analysis and isolation of the *trans*-Pt^{II}-modified PNA oligomers: LC–MS analysis of the crude products was carried out on a PE Sciex API 165 mass unit connected to a Jasco HPLC system. HPLC analysis was performed by using an Alltech Alltima C18 column (pore size 5 μm, diameter 4.6 mm, length 150 mm). A gradient elution (0 → 25% B in 30 min) was performed by building up a gradient starting with buffer A (1% TFA) and applying buffer B (1% TFA in 75% acetonitrile) with a flow rate of 1 mL min^{-1}. Compounds **5**, **6** and **7** were purified by reversed-phase HPLC on an Alltech Alltima C18 5μ (10 × 250 mm) column. A gradient elution (9 → 16% B in 40 min) was applied by building up a gradient starting with buffer A (1% TFA) and applying buffer B (1% TFA in 75% acetonitrile) with a flow rate of 5 mL min^{-1}. The purity of compounds **5**, **6** and **7** was established by LC–MS under the same conditions as used for analysis of the crude products.

ESI MS: **5**: m/z 2215 [M^+], 1108 [M^{2+}/2], 739 [M^{3+}/3]
6: m/z 1172 [M^{2+}/2], 782 [M^{3+}/3]
7: m/z 2741 [M^+], 1371 [M^{2+}/2], 914 [M^{3+}/3]

Cross-linking and isolation of the product: Compound **5** (2.572×10^{-8} mol) was treated with 5′d(GCGAATG) (**8**, 5.145×10^{-8} mol) in aqueous $NaClO_4$ (0.1M, 500 μL) and DMF (100 μL) at pH ~ 6.5 and at RT for 11 d. Compound **9** was purified by reversed-phase HPLC on an Alltech Alltima C18 5μ (4.6 × 150 mm) column. A gradient elution (0 → 23% B in 40 min) was performed by building up a gradient starting with buffer A (triethylammonium acetate (TEAA) 50 mM) and applying buffer B (50 mM TEAA in 75% acetonitrile) with a flow rate of 5 mL min^{-1}. MALDI-TOF MS: m/z: 4325 [M^+].

Analysis of the cross-linked product: MALDI-TOF MS spectra were obtained on a PE-Biosystems Voyager DE-PRO MALDI-TOF mass spectrometer equipped with delayed extraction and a reflector. Samples were irradiated with short pulses of a nitrogen laser emitting at 337 nm. The matrix used was 3-hydroxypicolinic acid. The matrix solution consisted of 3-HPA (3-hydroxypicolinic acid) (10 mg mL^{-1} in water/acetonitrile 1:1)/diammonium citrate (50 mg mL^{-1} in water) 8:1. The samples were diluted to 10 pmol μL^{-1} and mixed 1:1 with the matrix solution. 1 μL of this solution was put on the target and allowed to dry.

Chemical mapping of the cross-linked residue. A 10 μL-reaction mixture containing the cross-linked product **9** (1 pmol) (or single-stranded oligonucleotide **8**), $MgCl_2$ (10 mM), Tris.Cl (70 mM), pH 7.6, DTT (5 mM), ^{32}P-γATP (1μL, 10 mCi mL^{-1}; Amersham-Pharmacia Biotech) and T4 polynucleotide kinase (1 μL; New England Biolabs) was incubated for 1 h at 37 °C. Note that in the case of the cross-linked product **9**, only the 5′-end of the DNA strand is labeled whereas the PNA strand remains unmodified. The oligonucleotide **8** and product **9** with radio-labeled 5′-ends were purified on a denaturing 24% polyacrylamide gel. About half of each sample was treated with DMS, as described previously.[29, 30] The methylated G residues were then cleaved with hot piperidine.[29, 30] The remaining halves of the samples were treated with Fe^{II} and EDTA in the presence of hydrogen peroxide according to published procedures.[31] The cleavage products resulting from the DMS and Fe–EDTA treatments were then resolved on a denaturing 24% polyacrylamide gel (Figure 1). Gel data were analyzed by using a PhosphorImager (Molecular Dynamics) scanning.

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