Platinum(II)-Based Coordination Compounds as Nucleic Acid Labeling Reagents: Synthesis, Reactivity, and Applications in Hybridization Assays

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The synthesis, characterization, and molecular interactions of platinum(II) coordination compounds, which contain a distal nonradioactive reporter molecule, with mono- and polynucleotides are described. A [Pt(II)(en)($NH_2(CH_2)_6$NH-tBoc)Cl](NO_3) (en = ethylenediamine) entity has been coupled, after removal of the tBoc group, to a number of hapten and fluorophore molecules through succinimide derivatives. The influence of the various tethered reporter groups within these complexes on the reactivity towards guanosine 5′-monophosphate (5′-GMP), as a model for polynucleotide sequences, was investigated to shed light on the use of these reagents in hybridization assays. Reactivity turned out to be strongly dictated by the chemical nature of the distal reporter molecule present. At pH 7.0 the sequence of reactivity is cationic ≈ aromatic (stacking) > neutral > anionic; there is approximately an order of magnitude difference between the fastest reacting complex ($k = 10.2 \times 10^{-2}\,M^{-1}s^{-1}$) and the slowest reacting complex ($k = 0.93 \times 10^{-2}\,M^{-1}s^{-1}$) under these conditions. Platination of an oligodeoxynucleotide (30-mer), dsDNA, or an RNA transcript, shows that a Pt/nucleotide ratio between 1:10 and 1:20 (established by using flameless atomic absorption spectroscopy) results in probes with excellent hybridization characteristics. In terms of applicability and detection limits these platinated nucleic acid probes perform equally well compared to conventionally generated nucleic acid probes, that is, through enzymatic incorporation of covalently labeled nucleotide triphosphates. Applications of these reagents to in situ hybridization assays and gene expression profiling on microarrays illustrate the potential of these monofunctional binding platinum triamine compounds.

KEYWORDS:

fluorescent probes · guanosine · microarrays · nucleic acids · platinum

Introduction

The ability to detect nucleic acids is based on the extraordinary feature of DNA strand complementarity and the ability of single strands of nucleic acids to form double-stranded molecular hybrids in vitro. This hybridization phenomenon allows the specific detection of nucleic acids in biological samples like biological fluids, tissue sections, or cytological preparations on microscope slides, or following fixation on solid supports such as nylon membranes and glass. This procedure forms the basis of applications like Southern hybridization and in situ hybridization (ISH) that are used in many areas of research and clinical diagnosis. In the 1990s the emerging tools in the field of DNA microarray technologies enabled the generation of large amounts of data on many genes in a highly parallel manner. These arrays consist of a highly ordered matrix of thousands of different DNA sequences that can be used for gene expression profiling, comparative genomics, and genotyping.[1–4] Nucleic acid labeling, hybridization, and subsequent detection are indispensable parts of procedures necessary for gene identification and characterization.

During the last two decades nonradioactive nucleic acid labeling and detection systems have taken over from the safety,

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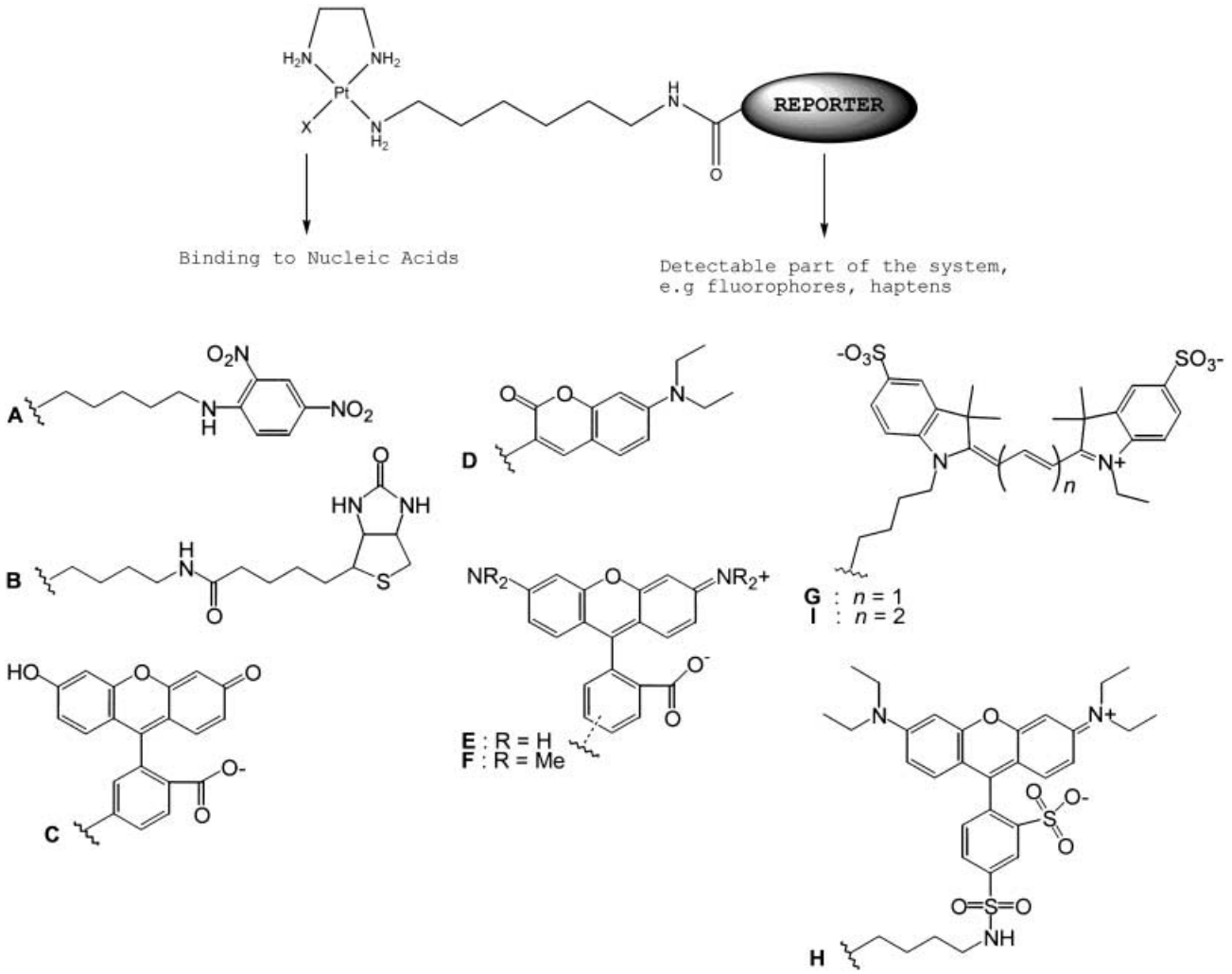
stability, disposal, and cost problems that are associated with radioisotope-based techniques. A comprehensive overview of all nonisotopic labeling methods, including both enzymatic and chemical modification of nucleic acid templates, has been presented by Kessler.[5] Kricka recently reviewed the last decade's developments in reporter chemistry and assay perspectives.[6] Enzyme-mediated labeling methods, for example, random priming, nick translation, and cDNA synthesis, are based on the incorporation of reporter molecules covalently attached to nucleotide triphosphate substrates in polymerization processes.[7] Several chemical labeling systems have been developed, ranging from post-synthetic modification of oligonucleotide sequences by phosphoramidite chemistry to photo-activated reagents such as psoralen.[8] Other covalent modifications can be achieved through transamination between cytidine derivatives and primary amines resulting in N^4-substituted cytidines or modification of C^5 of cytidine and uridine by an allylamine and subsequent condensation with activated esters of haptens.[9, 10]

It was reasoned that a platinum-based reagent could fulfill a pivotal role in chemical nucleic acid labeling and detection, as it would not be dependent on a priming event and the presence of a 3′-OH group. As the binding of such a metal complex is predominantly at the N^7 positions in guanine bases,[11] it was envisaged that there would be hardly any discriminatory effect between different nucleic acid backbones (RNA versus DNA, artificial sequences like PNA or LNA oligos), and also the length of the nucleic acid would not have a relatively major consequence for labeling, thereby providing a universal labeling system (ULS). The platinum reagents, which have been designed, synthesized, and evaluated for these purposes in labeling and detection of nucleic acids, are depicted in Scheme 1.[12–14]

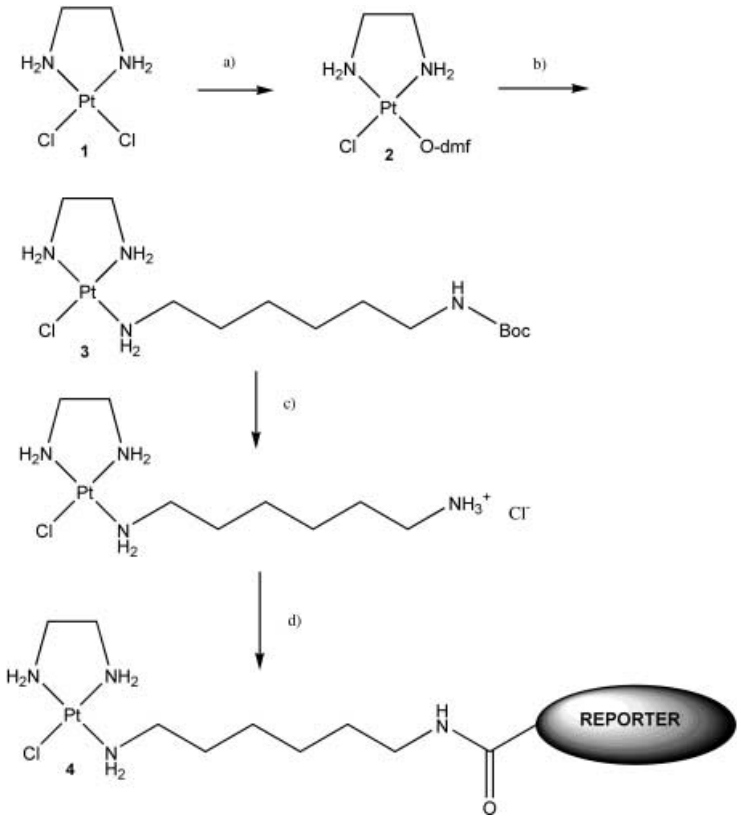
The general structure has similarities with often-studied monofunctional binding platinum reagents like [Pt(dien)Cl]Cl or $[Pt(NH_3)_3Cl]Cl$ (dien = diethylenetriamine). These compounds are frequently used as models for studies related to either kinetic or structural aspects on cisplatin – DNA interactions.[15–20] Relevant binding, structural, and stability parameters established for these complexes therefore provide insight to a certain extent into the platinum-based reagents shown in Scheme 1. It is generally agreed that the first binding step of platinum complexes towards nucleic acids is kinetically enhanced by the presence of the phosphate 5′ to the platination site.[21] However,



Scheme 1. *General structure of the platinum-based labeling reagents (upper part) and structures **A** – **I** of the reporting moieties described in this work (lower part); **A**: 2,4-dinitrophenyl, **B**: biotin, **C**: fluorescein, **D**: diethylaminocoumarine, **E**: carboxyrhodamine, **F**: rhodamine, **G**: cyanine3, **H**: lissamine, **I**: cyanine5.*

local factors can also contribute significantly to platination kinetics as exemplified in a number of papers.[22–24] From a DNA duplex stability point of view there are a number of studies indicating sequence-specific or local distortions as a consequence of such monofunctional platinum binding towards DNA.[25, 26] Others describe that DNA duplex stability of short oligo sequences is strongly affected by compounds of the type $[Pt(NH_3)_2(Ar\text{-}N)Cl]$, where Ar-*N* is an aromatic amine (e.g., 4-methylpyridine).[27] Later reports suggest that effects are more subtle and depend on the nature of Ar-*N* for instance.[28] For these reasons a primary amine was selected as the attachment point for the reporting entity and a chelating ethylenediamine ligand in the *trans* position to the entering nucleophiles within the nucleic acids. A conformational NMR study by van Garderen et al. on an *ss* and *ds* 9-mer showed that the distortion in the DNA helix by monofunctional binding is localized to the platinated base and 2 – 3 base pairs on its 5′ side.[29] Nevertheless, none of the Watson – Crick base pairs seemed to be (completely) disrupted.

The platinum-based nucleic acid probes have been shown to perform well in a series of (F)ISH and membrane-based applications.[13, 15, 30, 31] Until now, only little information has been available on the level of molecular interactions of these platinum complexes with the basic constituents of polynucleic acid sequences. The present paper reports on the synthesis of the reagents and subsequently describes the specific properties of these reporter – Pt structures, especially their interactions with (poly)nucleotides in order to provide a better understanding of the reaction profiles of these reagents.



Scheme 2. *Synthesis of the reporter – Pt compounds* ***4****. Reagents: a) 0.95 equiv. $AgNO_3$, DMF, RT, 16 h; b) 0.8 equiv. $NH_2(CH_2)_6NHBoc$, DMF, 40 °C, 16 h; c) 0.2 M HCl, 50 °C, 16 h; d) 0.8 equiv. reporter – NHS, RT, DMF/H_2O/200 mM NaCl, pH 8.0, 3 h. Boc, 1-butyloxycarbonyl; NHS,* N*-hydroxysuccinimide.*

Results and Discussion

Synthesis and characteristics of reporter – Pt compounds 4 A – I

An overview of the synthetic steps is depicted in Scheme 2. The starting material for the synthesis of the reporter – Pt complexes (**4**) is $[Pt(en)Cl_2]$ (**1**). Upon activating this complex with 0.95 equivalents of $AgNO_3$ in DMF the chloride ligand is displaced by a dmf ligand to afford **2** (^{195}Pt NMR: $\delta \approx -2071$ ppm). This activated complex is subsequently incubated with 0.8 equivalents of $NH_2(CH_2)_6NH$-*t*Boc[32] and after work-up provides **3** $[Pt(en)(NH_2(CH_2)_6NH\text{-}tBoc)Cl](NO_3)$ (^{195}Pt NMR: $\delta = -2632$ ppm) in an average yield of 74% and a purity > 90% (based on ^{1}H and ^{195}Pt NMR analysis). Compound **3** was subsequently deprotected by treatment in 200 mM HCl at 50 °C for 16 h. The formed $[Pt(en)(NH_2(CH_2)_6NH_3^+)Cl]Cl_2$ afforded a synthon, after pH adjustment, with an anchoring point for subsequent reporter – *N*-hydroxysuccinimide (NHS) conjugation. The generated reporter – Pt reagents (Table 1) could all be purified by either reversed-phase or ion-exchange chromatography and the desired labeling reagents

Table 1. *Some spectroscopic parameters of the reporter – Pt complexes (****4****) as structurally depicted in Scheme 1.*

No.	reporter (abbreviation)	$\lambda_{max,em}$ [nm] (λ_{exc} [nm])	$\lambda_{max,abs}$ [nm]	^{195}Pt NMR [ppm][i]
A	Dinitrophenyl (DNP)	n.a.	363[a]	− 2632[d]
B	Biotin (Bio)	n.a.	n.a.	− 3250[e]
C	Fluorescein (Flu)	517 (445)	495[b]	− 2618[f] − 2348[g]
D	Diethylaminocoumarin (DEAC)	476 (390)	421[a]	n.d.[h]
E	Carboxyrhodamine (CRho)	528 (460)	500[c]	n.d.[h]
F	Rhodamine (Rho)	578 (510)	552[c]	n.d.[h]
G	Cyanine 3 (Cy3)	566 (510)	550[c]	n.d.[h]
H	Lissamine (Liss)	591 (530)	569[c]	n.d.[h]
I	Cyanine 5 (Cy5)	666 (610)	648[c]	n.d.[h]

[a] In MeOH/H_2O (1:1, v/v). [b] In 10 mM TRIS pH 9.0. [c] In 10 mM TRIS pH 7.0. [d] In 10 mM HCl. [e] In H_2O. [f] In MeOH/20 mM NaCl/pH 4.0. [g] In 5 mM NaOD. [h] Not determined because of the low solubility of the reagent. [i] Relative to K_2PtCl_6 in water.

were obtained in chemical yields varying between 22 and 55%. The identities of the described complexes were confirmed by ^{1}H and ^{195}Pt NMR analysis, and the purities were verified by analytical reversed-phase chromatography: an average purity >85% was found (see Supporting Information). Some key structural and spectroscopic parameters of the compounds are listed in Table 1.

General physicochemical and reactivity properties of the reagents appear to be strongly dictated by the reporter molecule attached. In the case of Dinitrophenyl(DNP) – Pt (**4A**), the aromatic secondary amine at the dinitrophenyl group has a remarkable tendency to coordinate to the platinum metal at pH values above 4; in the ^{195}Pt NMR spectrum the resonance at $\delta = -2632$ ppm was shifted to $\delta = -2883$ ppm, indicative of a Pt – N_3Cl chromophore rearrangement into Pt – N_4. The latter product did not show any reactivity towards the N^7 position in guanosine 5′-monophosphate (5′-GMP) in comparison to the freshly prepared (Pt – N_3Cl surrounding) material. However, upon exposure of the Pt – N_4 adduct to acidic conditions (10 mM HCl) the complex reverted into its original Pt – N_3Cl state, which does platinate the N^7 position of guanine bases. The biotin(Bio) – Pt (**4B**) compound showed some notable chemistry as well, since after isolating the compound, ^{195}Pt NMR analysis indicated that the thioether moiety of the hapten structure participates in either intramolecular or intermolecular substitution of the chloride ligand. This stable macrochelate does, nevertheless, show reactivity towards the N^7 atom of 5′-GMP.

The complex containing the bifunctional reporter molecule fluorescein (**4C**)—bifunctional in the sense that it acts as a fluorophore, but can also serve as a hapten for antibody staining—required an expeditious route in order to sufficiently stabilize the reagent. As solubility improves at elevated pH values an alkaline solution (10 mM NaOH) was chosen. In this system the reagent initially functioned well in terms of reactivity towards nucleic acid templates. However, analytical reversed-phase chromatography indicated rapid (within days at 4 °C) rearrangements into additional species. The ^{195}Pt NMR spectrum had a single resonance at −2348 ppm (Figure 1), indicative of a Pt – N_3O surrounding,[33] most likely as a consequence of OH^- or carboxylate (present in the fluorophore) coordination under these conditions. In an attempt to achieve uniformity between the reporter – Pt complexes and to circumvent the potential inactivation by hydroxide coordination,[34] we chose to modify the solvent in which the fluorescein – Pt compound was kept. An aqueous methanolic solvent made slightly acidic by minute quantities of 0.1 M HCl was found to prevent ligand-exchange reactions, and therein the complex gave a ^{195}Pt NMR resonance at −2618 ppm (Figure 1) indicative of the desired Pt – N_3Cl surrounding. Solubility of the complex was reduced approximately tenfold to 0.5 mM, which is sufficient for labeling purposes. The presence of a second species in the analytical reversed-phase chromatogram (see Supporting Information) with a retention time of 16.41 min (versus the main product at 15.93 min) appears to be pH-dependent. The contribution of the faster running compound increases upon raising the pH to 5, whereas at pH 3 the contribution of the slower running compound gets close to 50%. MALDI-TOF analysis of these

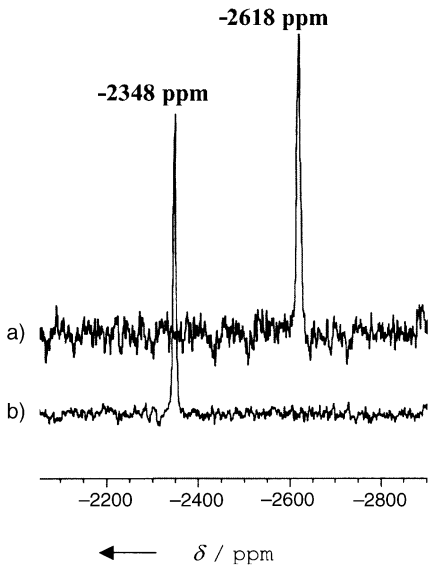


Figure 1. *^{195}Pt NMR resonances of compound **4C** in slightly acidic (a) and alkaline (b) media. a) 50% MeOH, 20 mM NaCl, pH 4.0; b) 10 mM NaOH, pH ≥ 10.0. Chemical shifts are in ppm relative to K_2PtCl_6.*

respective samples had *m/z* 763 $[M+H]^+$ (calcd.: 763) in both cases and no differences in fragmentation pattern. In combination with the previous observations this suggests that the observed species may well relate to a protonation/deprotonation effect of the carboxylate in the fluorescein, as pK_a values of such carboxylates are commonly in the range of 4.

Some general remarks can be made for the series of fluorescent reporting entities **4D** – **I**. Most of the seven complexes required an (inert) organic solvent for solubilization such as methanol or DMF at 25 – 50% v/v. In contrast to the observed coordination of donor groups of the three haptens described before, no such observations were made for the fluorophores. The spectroscopic properties, that is, the excitation and emission maxima, of the fluorophore – Pt compounds were hardly affected by the introduction of the platinum center. As the solubility of most of these fluorophore – Pt complexes is fairly low in comparison with the above-described haptens biotin and DNP, it was not possible to record ^{195}Pt NMR spectra. Nevertheless, analysis by reversed-phase chromatography proved a useful tool for assessing the integrity of the reagents. In terms of chemical/thermal compound stability, these reagents did not show detectable degradation upon prolonged (>2 weeks) exposure at 50 °C when dissolved in an optimized solvent system.

Reactivity of reporter – Pt complexes 4 towards guanosine 5′-monophosphate (5′-GMP) as monitored by ^{1}H NMR spectroscopy

The interaction of the described reporter – Pt complexes with nucleic acids is of vital importance in the probe labeling applications. To establish fundamental parameters of reaction kinetics and the influence of the tethered reporter molecule on the platination reaction, the interactions between a number of reporter – Pt complexes and 5′-GMP were studied. For this

purpose equimolar amounts of reporter–Pt were incubated under identical conditions, that is, at 2.5 mM, 50 °C, pD = 7.0, with 5′-GMP. The course of the reaction was visualized by ^{1}H NMR spectroscopy. Platination of the N^7 atom of 5′-GMP results in a typical downfield shift of the H8 proton and, to a lesser extent, the H1′ proton.[35, 36]

As an example, part of the aromatic region of the ^{1}H NMR spectra depicting the course of the reaction between DNP–Pt **4A** and 5′-GMP is shown in detail in Figure 2A. The upfield shifting of the phenyl protons upon product formation with 5′-GMP suggests a stacking interaction between the dinitrophenyl group with the purine 6-membered ring; a similar observation was made for the DEAC–Pt reagent **4D** upon binding to 5′-GMP. Since the H8 protons of platinated G bases are susceptible to exchange with deuterium, the relative integration of the H1′ resonance of the starting material versus the platinated product was used as a reliable measure for product formation.[37] Figure 2B shows the data of the several reporter–Pt:5′-GMP reactions studied and clearly shows significant differences in reaction kinetics between the various reporter molecules. The second-order rate constants k for these substitution reactions was determined by using Equation (1);[38]

$$kt = x/[a_o(a_o - x)] \quad (1)$$

In this equation x is the amount of [Pt(en)(reporter)(GMP-N^7)] and a_o is the initial concentration of [Pt(en)(reporter)Cl]. The rate constants as well as the calculated half-lives of the reactions are listed in Table 2, respectively.

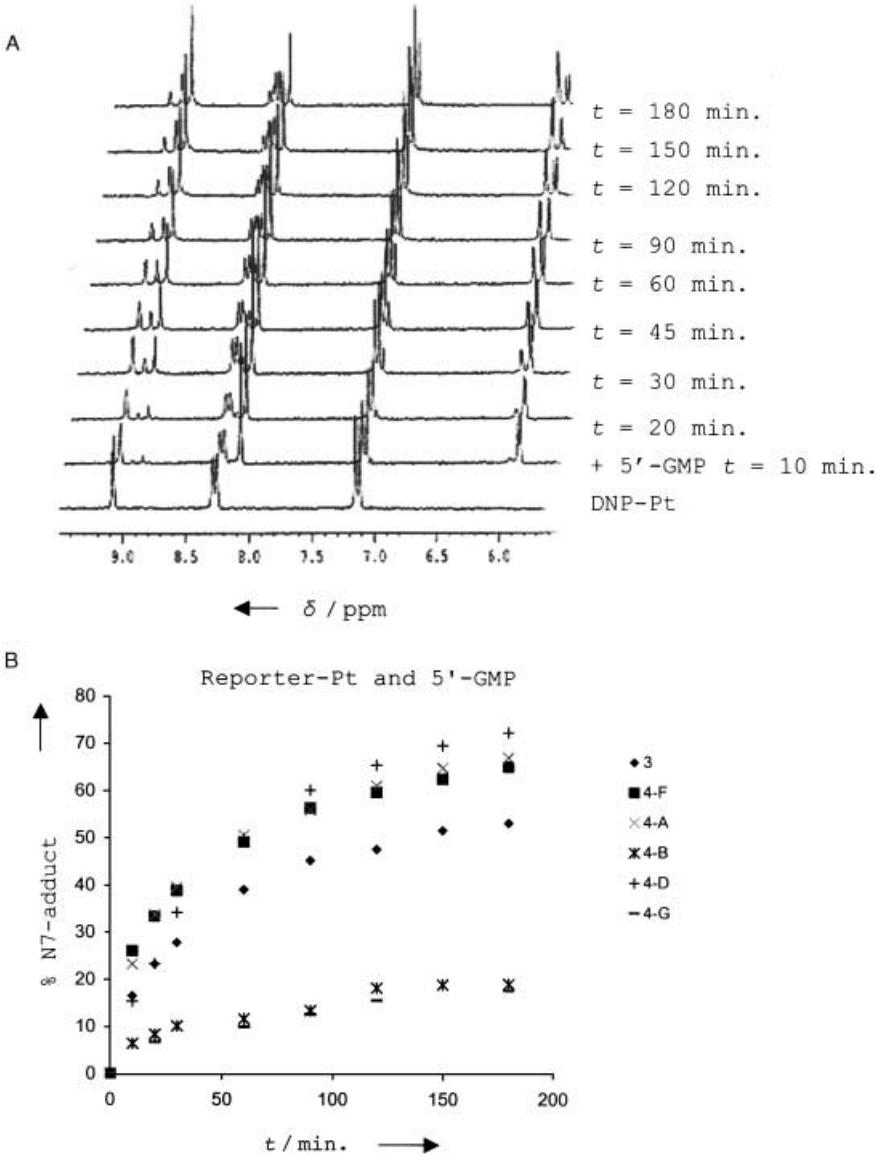


Figure 2. *Kinetic investigation of the reactivity of reporter–Pt complexes towards 5′-GMP. Part of the aromatic region of the ^{1}H NMR spectra of the DNP–Pt reagent with 1 equivalent of 5′-GMP showing the phenyl protons of DNP–Pt (**4A**) and the H8 and H1′ protons of 5′-GMP, in deuterated phosphate buffer at pD 7.0 at 50 °C, as a function of time (A); plots of the formation of N^7-platinated 5′-GMP for a series of reporter–Pt compounds as a function of time, derived from ^{1}H NMR data (B).*

The reactivity of [Pt(en)(NH$_2$(CH$_2$)$_6$NH-*t*Boc)Cl]$^+$ (**3**) with 5′-GMP will serve as a reference point ($t_{1/2}$ = 136 min) to allow a relative comparison of rate constants within this series. The *tert*-Boc protecting group can be categorized as a small, neutral, and—under the conditions applied—an inert functionality. The reactions of complexes **4A**, **4D**, and **4F** are clearly the fastest with half-lives between 65 and 85 min. As mentioned before, both the dinitrophenyl moiety in **4A** and the coumarin moiety in **4D** show upfield shifts of their respective aromatic resonances, indicating stacking interactions with the G base. This interaction is possibly attributed to the observed high reactivity towards 5′-GMP as compared to compound **3**. The rhodamine-containing complex **4F** is slightly less reactive than the previous complexes, but significantly faster than **3**.

Table 2. *Rate constants (k) and half-lives ($t_{1/2}$) for the reaction of [Pt(en)(reporter)Cl] with 5′-GMP at T = 50 °C at pD = 7.0.*

reporter–No.	k [10^{-3} M^{-1} s^{-1}]	$t_{1/2}$ [min]
DEAC–**4D**	102	65
DNP–**4A**	84.1	79
Rho–**4F**	78.5	85
*t*Boc–**3**	49.0	136
Bio–**4B**	10.6	628
Cy3–**4G**	9.25	720

The preferred binding affinity of platinum complexes towards the N^7 position in G bases is believed to be determined by a combination of three important factors: 1) electrostatic attrac-

tion between a negatively charged backbone or phosphate group and a positively charged platinum complex (all DNA bases), 2) the basicity of the N^7 position in guanine, and 3) the potentially hydrogen binding interactions between coordinated amines and the O^6 atom. The last two factors are the same for all platinum complexes under investigation in this study. The first of these factors, that is, the cationic nature of the rhodamine reporter in **4F**, which is also present in the green fluorescent derivative **4E** and far-red fluorescing lissamine – Pt **4H**, will most likely play the dominant role in overall reactivity for this set of complexes. At the other end of the reactivity spectrum are the cyanine-based reagents, exemplified by the Cy3 derivative in **4G**; the half-life for this reaction is 720 min, that is, an order of magnitude slower than the cationic and aromatic reporters described above. These remarkable differences in reactivity show that the affinity of this new series of platinum complexes for 5′-GMP, as a model for polynucleotide sequences, is strongly dictated by fundamental parameters in the ligand system, such as charge and base-association properties, whereas size and hydrophobic versus hydrophilic properties do not seem to influence the reaction to a large extent.

The observations for the biotin-containing complex **4B** can, strictly speaking, not be viewed along the same lines as the above-described series, since the starting complex has a Pt – N_3S surrounding in contrast to the Pt – N_3Cl surrounding in the other reactants. The substitution process of the *S*-bound part of biotin by the N^7 atom of 5′-GMP does, however, have similarities with the frequently studied rearrangement of [Pt(dien)(GSMe)] to [Pt(dien)(GMP-N^7)].[39, 40] Moreover and most remarkably, the half-life of this rearrangement as established in these studies was calculated to be 10.4 h (at 47 °C; other conditions being similar) which is very similar to the calculated value of 10.5 h for the binding of 5′-GMP to **4B**.

Quantification of polynucleotide modification by reporter –Pt compounds providing hybridization probes

Guided by the reactivity data, an empirical approach towards optimal platinum-modified nucleic acid probes for hybridization-based applications was followed. A series of variables affecting the platination reaction were evaluated, that is, reporter – Pt/nucleic acid (mass/mass) ratios, reaction temperature (50 – 90 °C), and reaction time (10 – 60 min). A typical nucleic acid labeling reaction was performed in the following manner: x μg ($x = 0.05 – 5$) of reporter – Pt complex was incubated with 1 μg of DNA or RNA; the platination reaction is subsequently quenched by the addition of an excess of sodium diethyldithiocarbamate (NaDDTC), which has ideal blocking characteristics for platinum compounds.[41] Unreacted and DDTC-inactivated reporter – Pt reagents were subsequently removed and the platinated nucleic acid sequence was isolated after a gel-filtration procedure. For hybridization purposes the probes are suspended in common hybridization buffers (e.g., formamide- or urea-containing buffers with probe concentrations varying from 25 ng μL^{-1} to 2.5 ng μL^{-1} for in situ applications and down to 25 pg μL^{-1} for membrane applications) and hybridized towards immobilized complementary target sequences, either in situ or on nylon membranes. Upon screening the above-mentioned reaction conditions the following optimal concentrations were established: $x = 0.3$, 0.4, 0.5, and 1 for the DNP-Pt, Carboxyrhodamine(Crho)-Pt, Bio-Pt, and Cyanine 5(Cy5)-Pt, respectively (based on dot-blot hybridizations onto serial dilutions for the haptens and FISH assays on metaphase spreads for the fluorophores). These findings and those attained with the other reagents in our series are well in concordance with the data obtained through the interaction with the model compound 5′-GMP.

Quantification of probe modification degree has been accomplished by determination of the platinum content by using flameless atomic absorption spectroscopy on a number of reporter – Pt-modified nucleic acid sequences.[42] The results for optimally modified dsDNA (Fish Sperm), an ODN 30-mer (d(GTATTAACTGTCAAAAGCCACTGTGTCCTG)), and an RNA fragment (1kB transcript) with both Bio – Pt and DNP – Pt are listed in Table 3. Modification degrees for both the fluorophore – Pt reagents Cy5 and CRho, as optimized in FISH assays, have been determined for the 2′-deoxyribonucleic acid sequences as well. There is neither a clear interlabel variation nor an internucleic acid variation; the Pt:nucleotide ratio differs between approximately 1:10 and 1:20. Higher nucleic acid modification degrees, that is, Pt:nucleotide ratios > 0.1, can be attained. However, this yields densely modified nucleic acid fragments with strongly altered physicochemical as well as hybridization properties (data not shown), which are currently being investigated in detail.

Table 3. *Pt/nucleotide ratio as determined by flameless atomic absorption spectroscopy. Maximum coefficient of variation per measurement is 1.5 %.*

	dsDNA	ODN[a] (30-mer)	RNA
DNP – Pt (**4A**)	0.05	0.07	0.06
Bio – Pt (**4B**)	0.08	0.10	0.08
Crho – Pt(**4E**)	0.09	0.10	n.d.[b]
Cy5 – Pt (**4I**)	0.11	0.11	n.d.[b]

[a] Oligodeoxynucleotide. [b] Not determined.

To semiquantitatively compare the modification degree as attained by our reporter – Pt reagents with enzymatically generated polynucleotide sequences, a serial dilution of these probes was spotted on a membrane and detected by using common immunochemical and chemiluminescent techniques. Figure 3 shows a serial dilution of such a spot-blot membrane in which biotinylated and DNP-modified mRNA are compared with enzymatically generated cDNA, both with biotin – dUTP and DNP – dUTP incorporation through oligo-dT-primed reverse transcriptase. The blot clearly shows that the specific activity of these probes is similar in terms of detection limits.

Applications of reporter – Pt reagents. Labeling and detection of DNA sequences in a FISH assay and gene expression profiling on a microarray platform

As example in which the above-described reagents have been successfully applied in labeling of whole chromosome painting (WCP) DNA probes[30] is given in Figure 4. The figure shows a

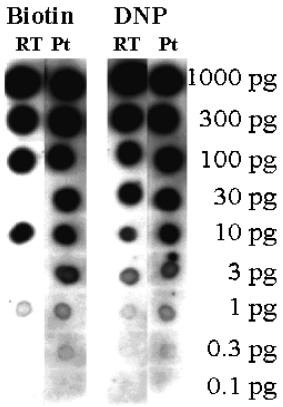


Figure 3. *A comparison of the labeling density of reporter – Pt complexes for mRNA (Pt lanes) with the labeling density achieved by reverse transcription (RT), by which labeled nucleotides are incorporated and labeled cDNA is generated (RT lanes). A dilution series (1000 pg down to 0.1 pg spotted nucleic acid) on positively charged nylon membranes is shown for DNP- and biotin-modified nucleic acid sequences. The haptens were detected by using anti-DNP – AP conjugates or Streptavidin – AP conjugates, respectively. Visualization was accomplished by chemiluminescence detection.*

twenty-four-color COBRA FISH on metaphase chromosomes from a chronic myeloid leukemic (CML) cell line.[30] COBRA FISH enables differential staining of all 24 human chromosomes by using 3 fluorochromes to ratio-label pairwise two sets of 12 WCPs, and a fourth fluorochrome is used to binary distinguish the two sets of ratiolabeled WCPs. The figure depicts a karyogram composed of the superimposed ratio-colors green (DEAC), red (Cy3), and blue (Cy5) on the foreground of the chromosomes and the pink shadow of the binary color (CRho) on the background of the odd-numbered chromosomes. This cell line contains a variety of numerical chromosome aberrations in addition to a few structural aberrations one of which is the t(9;22) (arrows), which is indicative for CML.[43]

DNA microarray technology has a significant impact on mRNA expression studies. This technology allows the monitoring of the whole genome of a certain organism on a DNA chip, thereby providing a tool for researchers to investigate the interactions among thousands of genes simultaneously. The DNA array consists of DNA sequences (PCR products or oligonucleotides) immobilized onto a solid surface (most commonly glass). The array is subsequently exposed to labeled nucleic acids, representing the cellular mRNA level, followed by the determination of the identity/

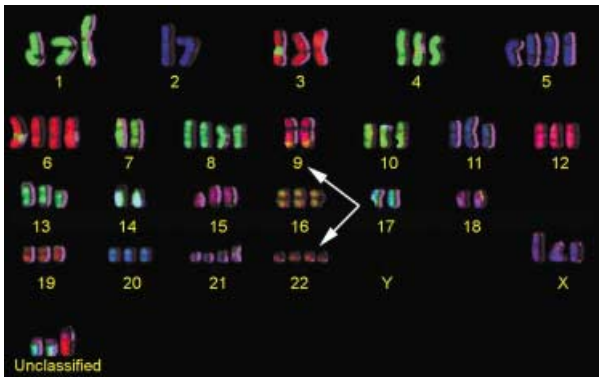


Figure 4. *Karyotyping by using COBRA-FISH in which WCPs are stained in a combined binary and ratio staining procedure using combinations of reporter – Pt reagents. A COBRA-FISH on a CML cell line is shown; the t(9,22) is indicative for CML, whereas a number of other chromosomal aberrations are apparent as well (courtesy of Prof. F. Mittelman and T. Fioretos MD PhD, Department of Clinical Genetics, University Hospital, Lund, Sweden).*

abundance of complementary sequences. Figure 5 shows the image of a microarray hybridization experiment, that is, the comparison of expression data between two murine tissues in a double label microarray experiment. Messenger RNAs isolated from both murine kidney and embryo were directly labeled with Cy3 – Pt and Cy5 – Pt, respectively. Subsequently, both labeled mRNAs were simultaneously hybridized onto a microarray containing ≈15 000 murine PCR-products. Hybridized mRNA is quantified by laser scanning. Fluorescence signals from the two different labeled mRNA molecules were distinguished by using two different emission filters. By scanning the arrays twice, once for Cy3 and once for Cy5, a composite image was generated in which the ratio of the two dyes, and hence, the ratio of mRNA molecules in the two tissues, was measured.

Conclusion

The synthesis and properties of a family of compounds with the general structure [Pt(en)($NH_2(CH_2)_6NH$ – reporter)Cl] (**4**), de-

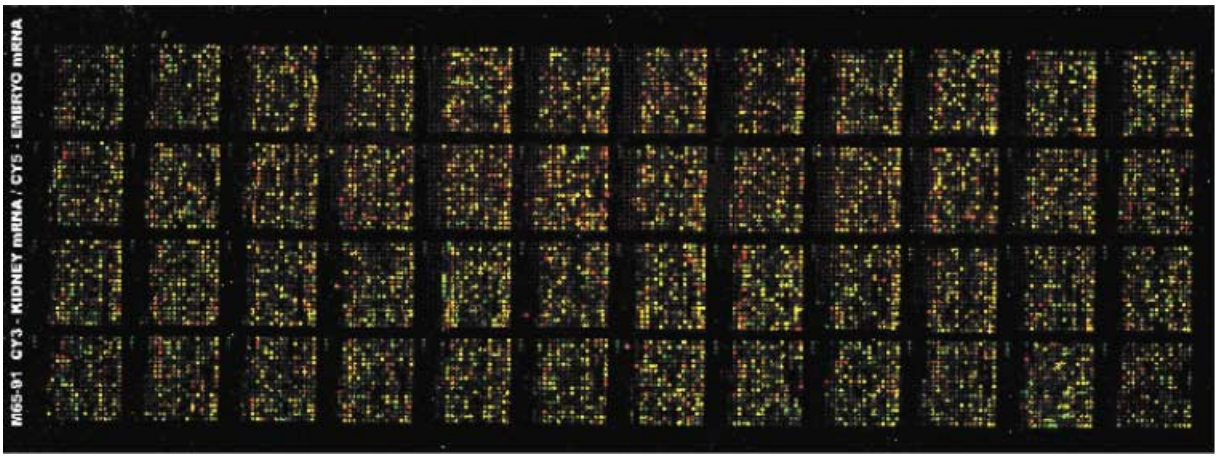


Figure 5. *Microarray image of a hybridization experiment with Cy3 – Pt-labeled mRNA from murine kidney and Cy5 – Pt-labeled mRNA from murine embryo. The labeled mRNA pools were mixed and hybridized to a 15 000 DNA microarray. Spots in green represent mRNAs in a relative higher abundance in the kidney; spots in red represent mRNAs in a relative higher abundance in embryo; yellow spots represent equal amounts of mRNA in both tissues.*

signed to act as monofunctional nucleic acid labeling reagent, have been described. The complexes were obtained in approximately 25 – 55 % overall chemical yield and were > 85 % pure by reversed-phase HPLC techniques. The complexes can be stored for prolonged periods without decomposition or ligand-exchange reactions by choosing the appropriate inert solvent systems and salt additives. The work has focused on the molecular interactions of the complexes with the constituents of DNA and RNA and has revealed the influence of the ligand structure, especially the tethered reporter moieties, on the binding behavior towards 5′-GMP and polynucleotide sequences. The reporter was shown to have a large effect on the rate constant of the substitution reaction of the chloride by the N^7 atom of 5′-GMP. Fundamental (chemical) parameters of the reporter molecules such as charge and base-association properties determine the reactivity profile in the following manner: aromatic ≈ cationic > neutral > anionic. The observations for 5′-GMP were translated to the modification of polynucleotide sequences and provided platinum-labeled nucleic acid hybridization probes. The optimal modification degree found for [Pt(en)($NH_2(CH_2)_6NH$ – reporter)] is between 1:10 – 1:20 (i.e., reporters:nucleotide) without significantly affecting the hybridization properties. Moreover, platinum-modified nucleic acid sequences behave similarly in hybridization applications to covalently (through C^5 on pyrimidines) modified sequences.

These platinum complexes have a general applicability in nucleic acid labeling procedures, as exemplified by FISH assays to detect chromosomal abnormalities in cells or metaphase spreads. Labeling cellular mRNA directly offers the possibility to profile gene-expression patterns, without the necessity of enzymatic target manipulation procedures. The chance on lower target bias increases by this approach, and the possibility to detect nonadenylated mRNA sequences, like in bacteria, becomes feasible.[44] Besides the nucleobase nitrogens as a binding site for platinum complexes, it is also possible to apply the same reagents for the labeling and detection of proteins through their Met or His residues. The platinum reagents as described in this work can therefore be applied to label crude biological samples (e.g., cell lysates, serum) as a whole and subsequently detect, through nucleic acid or antibody capture molecules on beads or arrays, the biomolecules of interest.

Experimental Section

Materials and methods:

Chemicals and biochemicals: [Pt(en)Cl_2] (**1**) was prepared according to an established procedure.[45] Mono-Boc-hexanediamine was prepared according to a slight adaptation of the procedure from Krapcho and Kuell.[32] Succinimidyl-6-(biotinamido)hexanoate was obtained from Pierce. 6-(2,4-Dinitrophenyl)aminohexanoic acid, succinimidyl ester (DNP-X, SE), 7-diethylaminocoumarin-3-carboxylic acid-SE (DEAC), 6-carboxyfluorescein-SE (Flu), 5-(6)-carboxyrhodamine-SE (CRho), 6-carboxytetramethylrhodamine-SE (Rho), and Rhodamine Red™-X,SE (Liss) were purchased from Molecular Probes. Cyanin3™- and Cyanine5™-monoreactive NHS dyes were purchased from Amersham Biosciences. 5′-GMP and fish sperm DNA were obtained from Sigma Chemical Co. The 30-mer d(GTATTAACTGTCAAAAGCCACTGTGTCCTG) was synthesized according to standard procedures and purified by ion-exchange chromatography. A 1065 nt RNA transcript was generated from a pGEMT control template by using a ribomax transcription kit from Promega.

Instruments and analytical methods: Reporter – Pt complexes were purified by reversed-phase chromatography using an ÄKTA FPLC system or an ÄKTA HPLC system. Source™ 15RPC (Amersham Biosciences) served as a reversed-phase stationary phase in either HR 16/10 columns for FPLC-based purifications, whereas a Phenomenex Luna, C18 (2) 5μ, 21.20 × 250 mm was used for the HPLC system. For analytical reversed-phase chromatography a Phenomenex Luna, C18 (2) 5μ, 4.6 (or 3.0) × 250 mm, was used. For desalting of certain products, Sep-Pak C18 (disposable) columns were used (Waters, WAT05190).

^{1}H and ^{195}Pt NMR spectra were recorded on a Bruker DPX 300 MHz spectrometer. ^{1}H chemical shifts in ppm were referenced to TMS. ^{195}Pt chemical shifts were referenced externally to K_2PtCl_6. Spectra were recorded at ambient temperature unless indicated otherwise. Absorbance characteristics in solution were determined on an Ultrospec 4000 UV/Vis spectrometer from Amersham Biosciences. Emission characteristics were determined on an LS45 luminescence spectrometer from Perkin – Elmer Instruments. Nucleic acid concentration determination was performed on an Ultrospec 2100 pro UV/Vis spectrometer from Amersham Biosciences.

Determination of platinum content was done by flameless atomic absorption spectroscopy (FAAS). The extent of platinum – DNA binding was determined by a Perkin – Elmer Atomic Absorption Spectrometer 3100 set to a slitband of 0.70 nm to monitor the Pt line at 265.9 nm. The limit of quantification was 1000 ng mL^{-1}. Deuterium background correction was used throughout analysis and the sample volume was between 0.020 mL and 0.060 mL. Furnace parameters were drying 120 °C/90 s, ashing 1300 °C/60 s, flushing 20 °C/15 s, and atomization at 2650 °C/5 s. Argon gas was used to purge the furnace.

Synthesis:

[Pt(II)(*N-tert*-Butoxycarbonyl-1,6-hexanediamine)(en)Cl](NO$_3$) (3): [Pt(en)Cl_2] (**1**) (500 mg, 1.53 mmol) was suspended in DMF (50 mL) and $AgNO_3$ (248 mg, 1.46 mmol, 0.95 equiv.) in DMF (10 mL) was added. The solution was stirred for 16 h at room temperature in the dark and filtered through membrane filters (0.2 μm) to remove AgCl. *N-tert*-Butoxycarbonyl-1,6-hexanediamine (315 mg, 0.8 equiv.) was dissolved in xylene (25 mL) and added to the [Pt(en)Cl(NO_3)] solution. The mixture was stirred overnight at 40 °C. Solvents were removed in vacuo and the remaining product was redissolved in a minute amount of Milli-Q water and stored overnight at 4 °C. Insoluble yellow particles of residual [Pt(en)Cl(NO_3)] were removed by filtration through membrane filters (1.0 μm). The clear filtrate was lyophilized to give the title compound (average yield 650 mg, 74 %) as a white fluffy powder. ^{1}H NMR (D_2O): $\delta = 3.11$ (2 H), 2.71 (4 H), 2.63 (2 H), 1.69 (2 H), 1.52 (2 H), 1.46 (9 H), 1.39 (4 H) ppm; ^{195}Pt-NMR: $\delta = -2625$ ppm.

General synthetic protocol for reporter – Pt complexes (4 A – I): In a typical experiment the following procedure was used: [Pt(en)(*N-tert*-Butoxycarbonyl-1,6-hexanediamine)Cl](NO$_3$) (**3**) (28 mg, 0.05 mmol) was dissolved in HCl (2 mL, 200 mM) and stirred overnight at 50 °C. The solution was neutralized by adding small quantities of 5 M NaOH. Sodium phosphate/NaCl buffer (1 mL, 250 mM, pH 8.0) was then added followed by Milli-Q water (2 mL). This solution was then mixed with reporter – NHS (0.04 mmol) dissolved in DMF (approximately 300 – 500 μL). The mixture was allowed to react for 3 – 16 h at room temperature. Solvents were removed in vacuo and the remaining product was redissolved in the minimal amount of loading buffer

and subsequently purified by reversed-phase chromatography. Elution buffers were subsequently removed by co-evaporation in vacuo; in some cases desalting over C18 Sep-Pak columns was required. Finally the products were lyophilized and subjected to analysis by NMR, UV/Vis, and fluorescence spectroscopy, respectively. MALDI-TOF analysis on compound **4D** was performed by Eurogentec s.a., Seraing, Belgium. Structural formulae of the compounds **4A–I** with the numbering used in the spectroscopic characterization can be found in the supporting information.

DNP–Pt {[Pt(en)(6-(((2,4-dinitrophenyl)amino-hexanoyl)amido)-hexylamine)Cl]Cl} (4A): Chemical yield: 53%. ^{1}H NMR (D_2O): $\delta =$ 1.29–1.80 (14H, m; $H_{e,f,g,j,k,l,m}$), 2.28 (2H, t; H_h), 2.66 (6H, m; $H_{n,o,o'}$), 3.14 (2H, t; H_d), 3.58 (2H, t; H_i), 7.20 (1H, d; H_c), 8.33 (1H, dd; H_b), 9.16 (1H, s; H_a) ppm; ^{195}Pt NMR: $\delta = -2632$ ppm; analytical reversed-phase chromatography: 100 mM TEAA pH 5.0/CH_3CN (9:1, v/v) was used as loading buffer and 100 mM TEAA pH 5.0/CH_3CN (3:7, v/v) as elution buffer. Retention time: 15.8 min.

Bio–Pt {[Pt(en)(6-(biotinamido)hexanoyl)amido)hexylamine)Cl]-NO_3} (4B): Chemical yield: 55%. ^{1}H NMR (D_2O): $\delta =$ 1.22–1.86 (20H, m; $H_{e,f,g,j,k,l,o,p,q,r}$), 2.20 (4H, m; $H_{h,m}$), 2.70 (8H, m; $H_{c,s,t,t'}$), 3.16 (4H, m; $H_{i,n}$), 3.58 (1H, m; H_d), 4.38 (1H, m; H_a), 4.69 (1H, m; H_b) ppm; ^{195}Pt NMR (D_2O): $\delta = -3254$ ppm; analytical reversed-phase chromatography: 0.05% TFA pH 2.5 as loading buffer and a 1:1 mixture of 0.05% TFA pH 2.5 and CH_3CN as elution buffer, retention time: 23.0 min.

FLU–Pt {[Pt(en)(6-(fluorescein-6-carboxamido)hexylamine)Cl]-NO_3} (4C): Chemical yield: 29%. ^{1}H NMR (D_2O/MeOD, 5:1 v/v): $\delta =$ 1.41 (4H, m; $H_{l,m}$), 1.59 (4H, m; $H_{k,n}$), 2.55 (6H, m; $H_{p,p',o}$), 3.39 (2H, t; H_j), 6.65 (4H; $H_{a,c,d,e}$), 7.15 (2H, d; $H_{b,f}$), 7.63 (1H, s; H_g), 7.95 (2H, dd; $H_{h,i}$) ppm; ^{195}Pt NMR (10 mM NaOH): $\delta = -2348$ ppm; ^{195}Pt NMR (20 mM NaCl/H_2O/MeOH 1:1, v/v at pH ≈ 4–5): $\delta = -2618$ ppm; $\lambda_{max,abs}$: 495 nm; $\lambda_{max,em}$: 510 nm; analytical reversed-phase chromatography: 100 mM TEAA pH 5.0/CH_3CN (9:1, v/v) was used as loading buffer and 100 mM TEAA pH 5.0/CH_3CN (3:7, v/v) as elution buffer. Retention time: 15.9 and 16.4 min; MS (MALDI-TOF): *m/z*: 763 (for both peaks).

DEAC–Pt {[Pt(en)(diethylaminocoumarin-3-(carbamido)hexylamine)Cl]NO_3} (4D): Chemical yield: 22%. ^{1}H NMR (D_2O): $\delta =$ 1.20 (6H, t; H_a), 1.42 (4H, m; $H_{i,j}$), 1.65 (4H, m; $H_{h,k}$), 2.60 (2H, m; H_l), 2.70 (4H, m; $H_{m,m'}$), 3.33 (2H, m; H_g), 4.48 (4H, q; H_b), 6.47 (1H, s; H_d), 6.76 (1H, dd; H_c), 7.47 (1H, d; H_e), 8.48 (1H, s; H_f) ppm; $\lambda_{max,abs}$: 421 nm; $\lambda_{max,em}$: 476 nm; analytical reversed-phase chromatography: 100 mM TEAA pH 5.0/CH_3CN (9:1, v/v) was used as loading buffer and 100 mM TEAA pH 5.0/CH_3CN (3:7, v/v) as elution buffer. Retention time: 21.8 min.

CRho–Pt {[Pt(en)5-(6-)-(carboxyrhodamine-5-(6-carboxamido)-hexylamine)Cl]NO_3} (4E): Chemical yield: 25%. ^{1}H NMR (D_2O/MeOD, 1:1 v/v): $\delta =$ 1.41 (4H, m; $H_{l,m}$), 1.65 (4H, m; $H_{k,n}$), 2.60 (6H, m; $H_{p,p',o}$), 3.43 (2H, t; H_j), 6.81 (4H, br s; $H_{a,b,d,e}$), 7.14 (2H, s; $H_{c,f}$), 7.63–8.45 (3H; $H_{g,i,h}$) ppm; $\lambda_{max,abs}$: 500 nm; $\lambda_{max,em}$: 528 nm; analytical reversed-phase chromatography: 100 mM TEAA pH 5.0/CH_3CN (9:1, v/v) as loading buffer and 100 mM TEAA pH 5.0/CH_3CN (3:7, v/v) as elution buffer. The two isomers are separately visible at retention times of 13.0 and 14.4 min.

Rho–Pt {[Pt(en)(6-(rhodamine)-6-carboxamido)hexylamine)Cl]-NO_3} (4F): Chemical yield: 31%. ^{1}H NMR (D_2O): $\delta =$ 1.43–1.69 (8H, m; $H_{m,n,o,p}$), 2.63 (6H, m; $H_{r,r',q}$), 3.18 (12H, s; $H_{a,f}$), 3.48 (2H, t; H_l), 6.58 (2H, s; $H_{d,e}$), 6.87(2H, d; $H_{b,g}$), 7.19 (2H, d; $H_{c,h}$), 7.87 (1H, s; H_i), 8.09 (2H, 2d; $H_{k,j}$) ppm; $\lambda_{max,abs}$: 554 nm; $\lambda_{max,em}$: 573 nm; analytical reversed-phase chromatography: 100 mM TEAA pH 5.0/CH_3CN (9:1, v/v) as loading buffer and 100 mM TEAA pH 5.0/CH_3CN (3:7, v/v) as elution buffer; the product elutes as a single peak at a retention time of 16.2 min.

CY3–Pt {[1-[Pt(en)-6-hexanediamine-6-hexanamido]-2-[3-[1-ethyl-1,3-dihydro-3,3-dimethyl-5-sulfo-2*H*-indol-2-ylidene]-1-propenyl]-3,3-dimethyl-5-sulfo-3*H*-indoliumchloride]} (4G): Chemical yield: 23%. ^{1}H NMR (D_2O): $\delta =$ 1.13–1.66 (14H, m; $H_{l,m,n,q,r,s,t}$), 1.48 (3H, t; H_a), 1.80 (12H, s; $H_{f,f',g,g'}$), 2.18 (2H, t; H_o), 2.58 (6H, m; $H_{v,v',u}$), 2.95 (2H, t; H_p), 4.18(4H, m; $H_{b,k}$), 6.43 (2H, d; $H_{w,y}$), 7.45 (2H, 2d; $H_{c,j}$), 7.91 (4H, m; $H_{d,e,h,i}$), 8.57 (1H, t; H_x) ppm; $\lambda_{max,abs}$: 550 nm; $\lambda_{max,em}$: 566 nm; analytical reversed-phase chromatography: 100 mM TEAA pH 5.0/CH_3CN (9:1, v/v) as loading buffer and 100 mM TEAA pH 5.0/CH_3CN (3:7, v/v) as elution buffer; the product elutes as a single peak at a retention time of 14.4 min.

Liss–Pt {[1-[Pt(en)-6-hexanediamine-6-hexanamido]-9-[2-carboxy-6-[[(2,5-dioxo-1-pyrrolidinyl)oxy]carbonyl]phenyl]-3,6-bis(-dimethylamino)chloride]nitrate}(4H): Chemical yield: 29%. ^{1}H NMR (D_2O/MeOD): $\delta =$ 1.26 (12H, br t; $H_{a,h}$), 1.27–1.54 (14H, m; $H_{o,p,q,t,u,v,w}$), 2.13 (2h, t; H_r), 2.57 (6H, m; $H_{x,y,y'}$), 3.09 (4H, m; $H_{s,n}$), 3.64 (8H, q; $H_{b,g}$), 6.96 (6H, m; $H_{c,d,e,f,i,j}$), 7.56 (1H, d; H_k), 8.19 (1H, d; H_l), 8.57 (1H, s; H_m) ppm; $\lambda_{max,abs}$: 570 nm; $\lambda_{max,em}$: 591 nm; analytical reversed-phase chromatography: 100 mM TEAA pH 5.0/CH_3CN (9:1, v/v) as loading buffer and 100 mM TEAA pH 5.0/CH_3CN (3:7, v/v) as elution buffer; the product elutes as a single peak at a retention time of 23.0 min.

Cy5–ULS {[1-[Pt(en)-6-hexanediamine-6-hexanamido]-2-[5-[1-ethyl-1,3-dihydro-3,3-dimethyl-5-sulfo-2*H*-indol-2-ylidene]-1,3-pentadienyl]-3,3-dimethyl-5-sulfo-3*H*-indoliumchloride]} (4I): Chemical yield: 38%. ^{1}H NMR (D_2O/DMF[D7], 1:1 v/v): $\delta =$ 1.20–1.85 (14H, m; $H_{l,m,n,q,r,s,t}$), 1.38 (3H, t; H_a), 1.73 (12H, s; $H_{f,f',g,g'}$), 2.18 (2H, t; H_o), 2.62 (6H, m; $H_{v,v',u}$), 2.99 (2H, t; H_p), 4.14 (4H, m; $H_{b,k}$), 6.37 (2H, dd; $H_{w,z}$), 6.66 (1H, dd; H_y), 7.37 (2H, 2d; $H_{c,j}$), 7.86 (4H, m; $H_{d,e,h,i}$), 8.18 (2H, m; $H_{x,z}$) ppm; $\lambda_{max,abs}$: 648 nm; $\lambda_{max,em}$: 655 nm; analytical reversed-phase chromatography: 100 mM TEAA pH 5.0/CH_3CN (9:1, v/v) as loading buffer and 100 mM TEAA pH 5.0/CH_3CN (3:7, v/v) as elution buffer; the product elutes as a single peak at a retention time of 16.2 min.

Reactivity towards 5′-guanosinemonophosphate (5′-GMP): Platination reactions were performed at a 2.5 mM concentration of both reactants in a deuterated 50 mM phosphate buffer pH 7.0 at 50 °C. Reactions were monitored by ^{1}H NMR spectroscopy. Calculations were performed by relative integration (estimated error 10%) of H1′ proton signals of both reaction product and starting material during the reaction.

Pt/nucleotide ratio determination on DNA, oligodeoxyribonucleotides, and RNA: In a typical platination experiment sonicated herring sperm DNA (20 μg) was labeled with DNP–Pt, CRho–Pt, Bio–Pt, and CY5–Pt in a mass:mass ratio of 1:0.3; 1:0.4; 1:0.5, and 1:1 in a total volume of 20 μL water. The input mass of reporter–Pt was doubled in the case of oligonucleotide labeling; other conditions were similar. The RNA transcript was platinated under similar conditions to the DNA by using Bio–Pt and DNP–Pt exclusively. Bio–Pt and DNP–Pt labeling reactions were performed for 30 min at 85 °C; CRho–Pt and Cy5–Pt labeling reactions were performed for 15 min at 65 °C. The platinated nucleic acid sequences were purified over a Sephadex-G50 spin column (Amersham Biosciences) and the volume made up to 1.0 mL by addition of demineralized water to provid a final nucleic acid concentration of 20 ng μL^{-1}. The samples were analyzed by FAAS as described above; a baseline signal or blank reaction was provided by samples in which equal amounts of reporter–Pt reagents were treated and purified without the presence of a nucleic acid template. Typically, the latter signal was 1% of the signal of the platinated nucleic acid.

DNA labeling and detection in a fluorescent in situ hybridization assay (FISH): The cell line LAMA-84 was obtained from The German Collection of Microrganisms and Cell Cultures (DSMZ).[46] The WCPs

were labeled in 4 separate labeling reactions. To this end, optimized amounts of each unlabeled (DOP – PCR-amplified) paint destined to get the same ULS label were mixed and reacted with the appropriate ULS label. After Qiagen column purification, the four labeled DNA solutions were mixed and a 3 × excess of human C_ot-1 DNA was added. After ethanol precipitation, the DNA was dissolved in the hybridization mixture. Subsequent FISH was performed as described previously,[30] with the exception that hybridization times were reduced from 5 to 2 days. For ratiolabeling, we used diethylaminocoumarin (DEAC) – ULS, Cy3 – ULS, and Cy5 – ULS. As binary ULS label, dGreen – ULS was used. The imaging and image processing of the WCP-COBRA-FISH was performed as described previously.[30]

RNA labeling with 4 A and 4 B versus reversed transcriptase (RT)-generated biotinylated and DNP-labeled cDNA and subsequent detection on a spot blot: Human placenta mRNA was obtained from Clontech. A 1-μg sample of the mRNA was labeled with Bio – Pt (**4 B**, 0.5 μg) and DNP – Pt (**4 A**, 0.3 μg) in a total volume of 20 μL DEPC treated demineralized water at 85 °C for 30 min. The reaction was subsequently quenched by the addition of NaDDTC solution (5 μL, 1 % m/v). The resulting solution contains 40 ng μL^{-1} of labeled mRNA. The enzymatically generated cDNA was prepared by starting with the same mRNA and by using a MICROMAX Labeling and Detection Kit (Perkin – Elmer Life Sciences Inc.) utilizing the AMV reversed transcriptase enzyme. The resulting solution contains 4 ng μL^{-4} of labeled cDNA. Serial dilutions of these labeled nucleic acid solutions were prepared from 1000 pg to 0.1 pg and spotted on nylon membranes according to standard procedures. Detection was performed by chemiluminescence by using anti-DNP – AP or Streptavidin – AP conjugates with reagents for detection from the MICROMAX Labeling and Detection Kit (Perkin – Elmer Life Sciences Inc.) according to the manufacturer's instructions.

Microarray hybridization and mRNA labeling with Cy3 – Pt and Cy5 – Pt: Both 1-μg samples of mRNA isolated from murine kidney and murine embryo were directly labeled with Cy3 – Pt (**4 G**) and Cy5 – Pt (**4 I**), respectively. Labeled mRNA was purified over a Chromaspin TE-30 column (Clontech) and subsequently washed and concentrated on a Microcon-YT 30 (Millipore) filter. Subsequently, both labeled mRNAs were hybridized on a microarray containing over 15 000 murine PCR-products (the National Institute of Ageing murine cDNA library, NIA/NIH, Bethesda, USA). Hybridization was performed at 42 °C in a hybridization cassette (Telechem International) under a coverslip (Lifterslip, Erie Scientific) in a volume of 40 μL.[47] The presence of bound mRNA was detected by fluorescence following laser excitation. Signals from the two different labeled mRNA molecules were distinguished by using two different emission filters in a ScanArray 4000 microarray scanner (Perkin – Elmer). By scanning the arrays twice, once for Cy3 and once for Cy5, a composite image was generated. Furthermore, the fluorescent signals were quantified by using the Imagene 5.0 software package (Biodiscovery) and used for measuring ratios of mRNA molecules in the two tissues.

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[46] Information on this cell line can be obtained at http://www.dsmz.de.
[47] See also http://microarrays.nki.nl.

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