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Effect of Thioethers on DNA Platination by *trans*-Platinum Complexes

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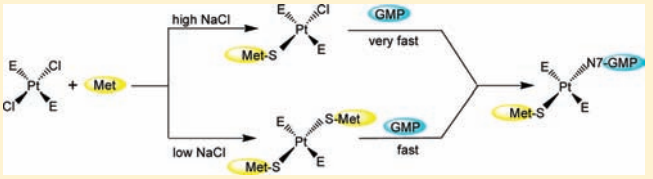
Supporting Information

ABSTRACT: Increasing evidence indicates that sulfur-containing molecules can play important roles in the activity of platinum anticancer drugs. Although nuclear DNA is retained to be the ultimate target, these platinum compounds can readily react with a variety of other substrates containing a soft donor atom, such as proteins, peptides, and low molecular weight biomolecules, before reaching DNA. In a recent study it was demonstrated that the DNA platination rate of a trans-geometry antitumor drug was dramatically enhanced by methionine binding, thus suggesting that the thioether could serve as a catalyst for DNA platination. In this work we performed detailed studies on the reactions of a widely investigated and very promising *trans*-platinum complex having two iminoethers and two chlorido ligands, *trans-EE*, with methionine (Met) and guanosine 5′-monophosphate (GMP). The results show that in the reaction of *trans-EE* with methionine the bisadduct is the dominant species in the early stage of the reaction. The reaction is also influenced by chloride concentration: at low NaCl the bis-methionine adduct is formed in preference, whereas the monoadduct is favored at high NaCl concentration. Not only the monomethionine complex, *trans*-PtCl(*E*-iminoether)$_2$(AcMet), but also the bis-methionine adduct, *trans*-Pt(*E*-iminoether)$_2$(AcMet)$_2$, which has already lost both leaving chlorides, can react with GMP to form the ternary platinum complex *trans*-Pt(*E*-iminoether)$_2$(AcMet)(GMP). The latter reaction discloses the possibility of direct coordination to DNA of a platinum−protein adduct, in which the two carrier ligands remain intact; this is not the case of cis-oriented platinum complexes, like cisplatin, for which formation of a ternary complex is usually accompanied by loss of at least one carrier ligand. Interestingly, isomerization from S to N coordination of one methionine takes place in the bis-methionine complex at neutral pH, while the monoadduct appears to be stable. The shift from S to N coordination of one methionine in the *trans*-bis-methionine adduct can easily account for the obtainment of the cis isomer in the bis-chelated Pt(Met-S,N)$_2$ end product.

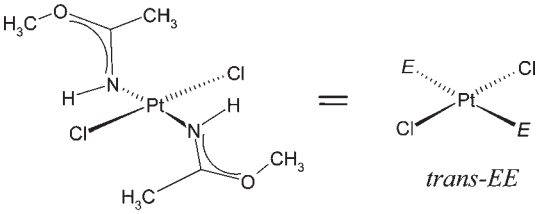


INTRODUCTION

Cisplatin (*cis*-[PtCl$_2$(NH$_3$)$_2$], *cis*-DDP) is widely used in the treatment of testicular, ovarian, and other tumors.[1,2] However, application is limited by side effects and acquired or intrinsic resistance.[3,4] Intensive efforts have been devoted to the discovery of platinum anticancer analogues, and two of them, carboplatin and oxaliplatin, have successfully reached the status of widely clinical use showing reduced toxicity and/or better efficacy.[5] Early structure/activity relationships suggested that the cis geometry is required for antitumor activity of platinum complexes, while trans compounds are inactive. However, in recent years, several platinum compounds with trans geometry have exhibited remarkable antitumor properties.[6,7] Interestingly, the *trans*-iminoether complex (*trans*-PtCl$_2$(*E*-iminoether)$_2$, *trans-EE*) demonstrates higher cytotoxicity than its cis isomer and is effective in several *cis*-DDP-resistant tumor cell lines (Scheme 1).[7,8]

It has been generally accepted that the cytotoxicity of platinum compounds is caused by DNA platination and eventually induced cell apoptosis.[2] However, a number of proteins get involved in the mechanism of platinum drugs, both at the level of drug transport and at cellular processing of the ultimate lesion(s) (including platinum inactivation and efflux and DNA repair).[9−12] Many investigations have been carried out for

Scheme 1. Sketched Structure of *trans*-PtCl$_2$(*E*-iminoether)$_2$ (*trans-EE*)



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elucidating how platinum drugs can survive attack of S-donor compounds and bind to DNA, since sulfur ligands have considerably higher affinity toward platinum than N7 of DNA purines.[13,14] On the other hand, it has been found that an elevated level of GSH can enhance cell sensitivity to cisplatin by up-regulating the copper transporter protein hCtr1.[15] hCTR1 is a membrane protein containing extracellular methionine rich motifs and can facilitate platinum transfer through the cell membrane, although in vitro experiments show carrier ligands loss depending upon the reaction time and solution conditions.[10,16−18] In an attempt to elucidate the detailed mechanisms of these processes, reactions of *cis*-DDP with sulfur-containing model compounds have been extensively investigated using, for example, synthetic peptides,[17,19] glutathione,[20−22] L-methionine,[23−26] *N*-acetyl-L-methionine,[23,27] and thiourea.[22]

Unlike *cis*-DDP, very few studies have been performed on active trans-geometry platinum complexes. In one of these studies it has been shown that methionine of cytochrome *c* is the primary binding site for *trans-EE*.[28] It has also been found that the histone protein H1 can bind to the *trans-EE*/DNA monoadduct and subsequently interfere with DNA repair by NER,[29] which further supports the significance of protein binding in the biological activity of trans-geometry platinum drugs. Unlike H1, HMG-domain-containing proteins, which bind to cisplatin-modified DNA and prevent DNA repair, do not recognize *trans-EE*/DNA adducts. The latter result highlights the fundamentally different DNA-binding motifs of *trans-EE* relative to *cis*-DDP, resulting in an overall different pharmacological activity. The different coordination geometries and divergent DNA binding modes of *trans-EE* and *cis*-DDP can prelude to a different mode of protein interaction for the two compounds. Thus, better knowledge of the mode of interaction of *trans*-platinum complexes with platinophiles and proteins could serve to better understand the differences between the two types of active antitumor compounds.

Recently, we discovered that binding of methionine to *trans-EE* could enhance significantly the DNA platination rate, suggesting a possible role of proteins in the activation of *trans*-platinum drugs.[30] Moreover, the binding of *trans-EE* to methionine residues of proteins was detected in living cells (*E. coli*), supporting the biological relevance of this process. Additionally, it has been shown that binding of methionine to *cis*-$[PtCl(NH_3)_2(pyridine)]^+$ can lead this monofunctional platinum antitumor agent to interact with DNA similarly to a *trans*-platinum substrate.[31] Here, we report a detailed investigation showing how L-methionine (Met) and *N*-acetyl-L-methionine (AcMet) can modulate the interaction of *trans-EE* with nucleotides. The reaction was monitored by NMR, and the product compositions were confirmed by electrospray mass spectrometry (ESI-MS). The effect of chloride concentration on the reaction between *trans-EE* and methionine was also investigated. Furthermore, we observed that the bis-methionine adduct *trans*-Pt(*E*-iminoether)$_2$(Met)$_2$ can undergo at neutral pH a fast rearrangement from S to N coordination of one methionine which can easily explain formation of a *cis*-Pt(Met-N,S)$_2$ end product.

EXPERIMENTAL SECTION

Materials. *trans-EE* was prepared by published procedures.[32] GMP (guanosine 5′-monophosphate), Met (L-methionine), and AcMet (*N*-acetyl-L-methionine) were purchased from Sangon Co., and the purity was checked by HPLC and ^{1}H NMR spectra prior to use (>95%). ^{15}N,^{13}C-Labeled methionine was purchased from Spectra2000 Srl. D_2O was purchased from Aldrich. The chemicals used for HPLC purification and sample preparation were of high purity and used without further purification; they include acetonitrile, triethylammonium acetate buffer (made from equimolar amounts of triethylamine and acetic acid), sodium hydrogen phosphate, NaCl, NaOH, and $NaClO_4$.

Sample Preparations. Stock solutions of *trans-EE* were prepared by dissolving the platinum complex in either H_2O or D_2O at a concentration of 0.8 mM and stored at −20 °C. AcMet, Met, and GMP solutions were prepared by directly dissolving a weighted amount of the compounds into a given volume of H_2O or D_2O. pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter connected to a Sentron Red-line pH probe. No correction for the deuterium effect on the pH reading was applied. Unless stated otherwise, no buffer was used and the pH was varied within 0.2 unit during the reactions. All reactions were carried out in 20 mM $NaClO_4$ at 25 °C in the dark.

NMR Spectroscopy Experiments. NMR spectra were recorded at 25 °C on a Bruker Avance 600 UltraShield Plus magnet using a triple-resonance (TXI) probehead and on Bruker Avance 300 UltraShield and Bruker DRX 400 magnets using broad-band inverse (BBI) probeheads. Each instrument was equipped with pulsed field gradients along the *z* axis. NMR spectra were recorded on samples dissolved in either H_2O or D_2O, and the pH was adjusted accordingly with NaOH for experiments aiming to detect exchangeable protons or NaOD for experiments detecting only nonexchangeable protons. The presaturation pulse sequence was used for suppression of residual HDO signal. A relaxation delay of 2 s was applied. The chemical shifts are referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) through the HDO resonance, in which the temperature effect was calibrated.[33] A small amount of CH_3OD was also added to the reaction system as an internal reference for chemical shifts and peak intensities.

2D ^{1}H,^{15}N, and ^{1}H,^{13}C HSQC, ^{1}H,^{13}C HSQC-TOCSY, and ^{1}H,^{13}C HMBC spectra were used to assign Met resonances both in the absence and in the presence of the platinum complex. 2D ^{1}H,^{195}Pt HSQC experiments were recorded using a fixed spectral width of 1000 ppm in the ^{195}Pt dimension and different values of transmitter frequency such as to scan a ^{195}Pt spectral region from 0 to −5000 ppm. The INEPT delay was set to an optimized value of 4.17 ms (corresponding to a $J_{Pt,H}$ coupling constant of 60 Hz). The ^{195}Pt resonance of Na_2PtCl_6 (0 ppm) was used to reference ^{195}Pt chemical shifts.

Mass Spectra. Mass spectrometric measurements were performed on a Thermo Xcalibur 2.0 instrument using a Thermo LTQ linear ion trap mass spectrometry (Thermo Fisher, San Jose, CA). Lyophilized samples were dissolved in 50:50 water/methanol solvent containing 0.1% formic acid. Samples were directly infused at a flow rate of 3 $\mu L \cdot min^{-1}$ and analyzed in positive mode, with a spray voltage of 1.8 kV and capillary voltage of 32 V. The capillary temperature was kept at 200 °C.

RESULTS

Reactions between *trans-EE* and AcMet or Met: Formation of a Bis Adduct Already in the Early Stage of the Reaction. The reactions between *trans-EE* and AcMet or Met were monitored by 1D ^{1}H NMR spectra (Figure 1). In principle, the reactions could be monitored from the change in intensity of the Me signal of methionine and the C-Me and the O-Me peaks of *trans-EE*. However, we could rely only upon the *trans-EE* methoxy peaks since the methionine Me and the *trans-EE* C-Me signals overlap. Using an excess of AcMet or Met (1:2.5 molar ratio), one peak, **H**, was rather intense already in the early stage of the reaction and increased with time (Figure 1a). The ESI-MS spectrum confirmed that **H** is the

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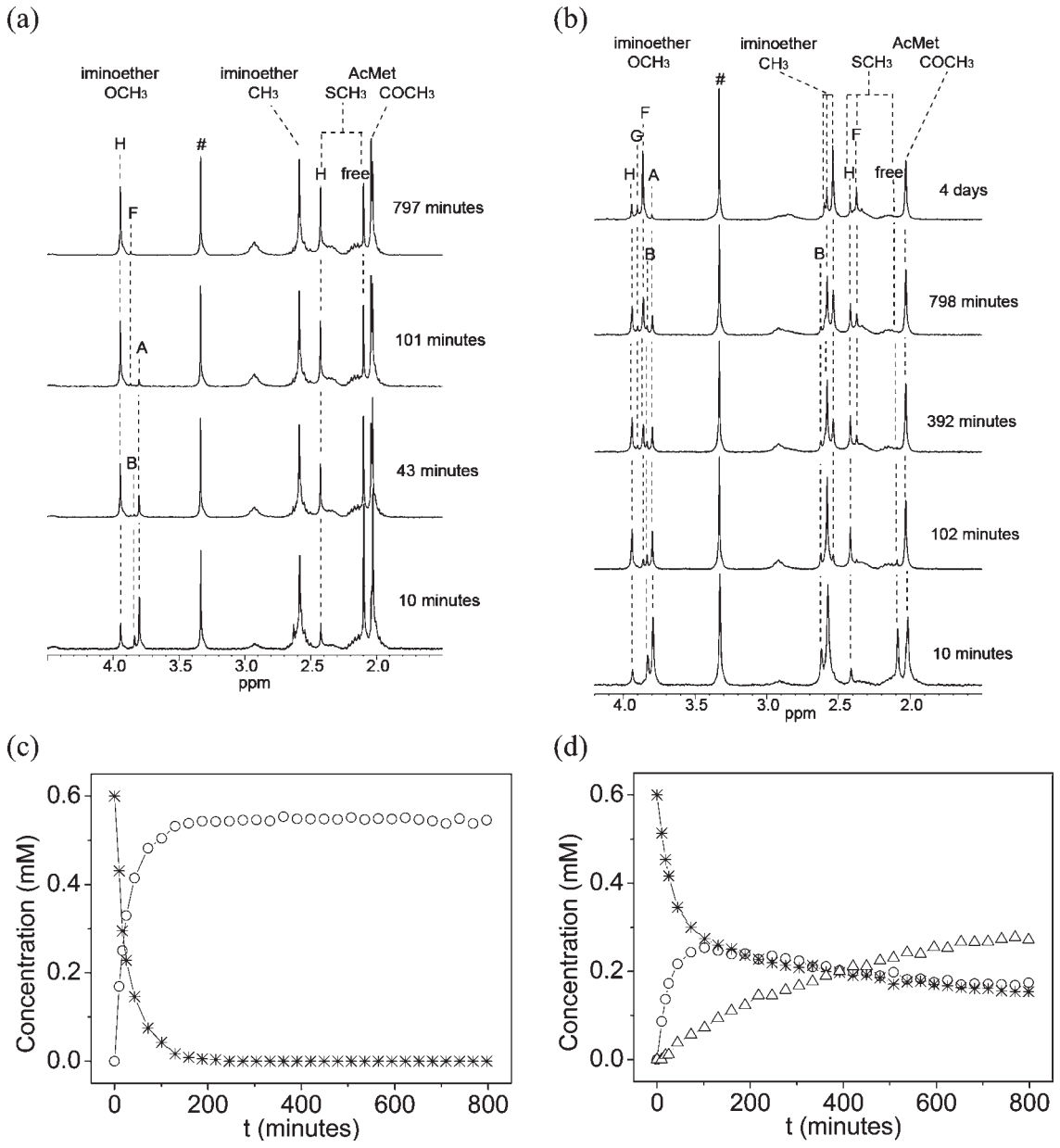

Figure 1. (a and b) 1D ^{1}H NMR spectra of the reaction between *trans-EE* and AcMet performed at 25 °C and in 20 mM $NaClO_4$. Reaction time is given on each spectrum. The resonance assignment is given on the top of the spectra and includes OCH_3 and CH_3 from the iminoether ligand and SCH_3 and $COCH_3$ from AcMet. The letters on the spectra indicate the species of Scheme 2 originating the signal. The symbol # denotes the CH_3OD added as reference: (a) [*trans-EE*]:[AcMet] = 1:2.5, pH 2.8 and (b) [*trans-EE*]:[AcMet] = 1:1, pH 3.1. (c) Plot of the concentrations of the starting substrates (**A** + **B**) and of the bisadduct (**H**, practically the exclusive reaction product in excess of AcMet) as a function of time for the reaction reported in a. (d) Plot of the concentrations of the starting substrates (**A** + **B**), of the bisadduct (**H**), and of the monoadducts (**F** + **G**) as a function of time for the reaction reported in b. Symbols denote the reactants (**A** + **B**) (*), the bis-methionine adduct (**H**) (○), and the monoadducts (**F** + **G**) (△).

bisadduct *trans*-Pt(*E*-iminoether)$_2$(AcMet)$_2$ (Figure 2a). In addition to **H**, another peak, **F**, was present throughout the reaction course and had a very small intensity (Figure 1a). The ESI-MS identified this peak as the monoadduct *trans*-PtCl(*E*-iminoether)$_2$-(AcMet) (Figure 2b). It is surprising that the bisadduct (**H**) forms so quickly while the monoadduct can hardly be seen even in the very early stage of the reaction. The plot of Figure 1c shows the decrease of the starting substrate (**A** + **B**) and increase of the bisadduct (**H**) as a function of time (the peak of monoadduct, **F**, was too small to be integrated with accuracy). Figure 1b shows the progress of a similar reaction performed at a 1:1 molar ratio. **H** is still the major product in the initial stage of the reaction but gradually converts to **F**. Conversion of **H** into **F** is clearly evident after about 2 h reaction time when the free AcMet is nearly completely consumed (Figure 1d). Since formation of the bisadduct necessarily requires preliminary formation of the monoadduct, it can be concluded that entering of the second molecule of methionine is considerably faster than coordination of the first molecule so that **F** is converted to **H** very rapidly as long as free AcMet is present. Consequently, in the presence of excess AcMet only the bisadduct **H** is detected (Figure 1a). In contrast, in the case of a 1:1 ratio, after consumption of free AcMet and formation of

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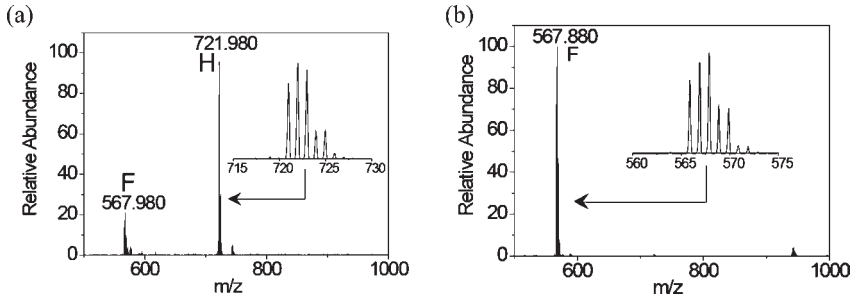
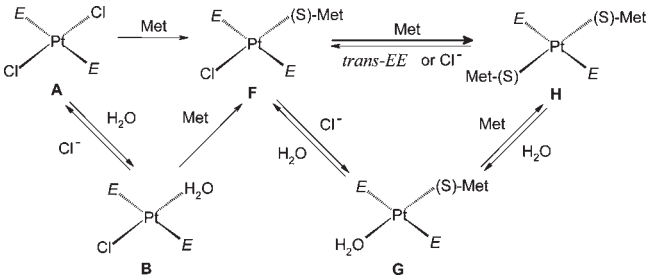


Figure 2. ESI-MS spectra of the products obtained in the reaction between *trans-EE* and AcMet. Spectra were recorded after 24 h reaction time: (a) [*trans-EE*]:[AcMet] = 1:2.5 and (b) [*trans-EE*]:[AcMet] = 1:1. Insertions show the expanded region of major peaks (**H** and **F**). Note that the peak intensities are not proportional to the concentrations due to the easier ionization of **F** over **H**.

Scheme 2. Reaction between *trans-EE* and Met



one-half the stoichiometric amount of the bisadduct **H**, free *trans-EE* can react with **H** forming the monoadduct **F**, which becomes the dominant species at long reaction time (Figure 1b and 1d). It was observed that even performing the reaction in excess *trans-EE* the bisadduct is still the dominant species in the initial stage of the reaction (spectra not shown). The different species formed in the reaction of *trans-EE* with AcMet or Met are shown in Scheme 2.

The reactions were also carried out in the presence of 100 mM NaCl in order to study the effect of the high NaCl concentration present in extracellular environment (Figure 3). Comparison between Figure 3a and Figure 1a (both experiments performed in the presence of excess AcMet) shows that unlike the case of the reaction performed in the absence of NaCl in the reaction performed in the presence of 100 mM NaCl the monoadduct **F** is a major component throughout the reaction course. Moreover, by comparing Figure 3b with Figure 1b (both experiments performed in a 1:1 *trans-EE*:AcMet molar ratio), it appears that although nearly pure **F** is the final product in both cases only in the presence of 100 mM NaCl this species is practically the only reaction product throughout the whole reaction course. In contrast, in the absence of added NaCl the bisadduct **H** dominates in the early stage of the reaction.

In the absence of added NaCl (Figure 1b), another minor peak (**G**) is observed which is assigned to the hydrolyzed monoadduct *trans*-[Pt(*E*-iminoether)$_2$(AcMet)(H$_2$O)]$^+$. This assignment is supported by the observation that addition of NaCl converts **G** into **F** (the chloro species).[34] Of course, peak **G** does not form in the experiment performed at 100 mM NaCl (Figure 3b). In the case of Figure 3b, in which the bisadduct **H** is practically absent and the monoadduct **F** is the nearly exclusive reaction product, by applying the conditions of second order (equal concentrations of the two reactants), it is possible to estimate the rate constant from the plot of $1/[trans\text{-}EE]$ against time. A reasonable straight line with slope 0.26 ± 0.01 M^{-1} s^{-1} (corresponding to a second-order rate constant) is obtained (Figure S1, Supporting Information).

Reaction of *trans-EE* with GMP in the Presence of Methionine. In a previous study it was shown that the ternary complex was the major product in the reaction of *trans-EE* with AcMet and GMP at an equal molar ratio.[30] Moreover, the overall rate of GMP platination depends upon the presence or absence of methionine, and as an example, in Figure S2, Supporting Information, is shown the decrease of free GMP as a function of time in a sample containing only *trans-EE* and GMP (1:1 molar ratio) or containing *trans-EE* and GMP in addition to Met (1:1:1 molar ratio). A similar effect was also observed in the reaction with excess GMP, where the ternary complex **J** remains one of the major products for the first 12 h to be slowly converted to the bis-GMP complex **E** only for longer reaction times. A similar behavior has also been observed using AcMet in place of Met. The faster GMP platination in the presence of Met (or AcMet) can be accounted for by an additional platination pathway (**F** to **J**) relative to direct GMP platination as illustrated in Scheme S1, Supporting Information.

Reaction of *trans-EE*/AcMet Complexes with GMP: Also the Bis Adduct with AcMet Can React with GMP. It was shown in a previous study that *trans-EE* reacts faster with methionine than with GMP and that formation of a mono-*trans-EE*/methionine complex can significantly enhance the DNA platination rate (brief summary given in the previous paragraph).[30] Here, we show that also the bisadduct *trans*-Pt(*E*-iminoether)$_2$(AcMet)$_2$ (**H**), which has already lost both leaving chlorides, can react with GMP in acidic conditions (Figure 4). One of the two AcMet molecules is substituted by GMP, and a ternary complex, *trans*-Pt(*E*-iminoether)$_2$(AcMet)(GMP) (**J**), is formed. **J** is the only ternary product throughout the reaction of **H** with GMP, and the reaction appears to be of equilibrium (no further decrease of H after 2 h reaction) (Scheme 3). Since the experiment (0.6 mM *trans*-Pt(*E*-iminoether)$_2$(AcMet)$_2$, 2.0 mM GMP, and 1.5 mM AcMet) was performed in the presence of excess GMP and AcMet, the forward reaction can be considered pseudo-first-order in *trans*-Pt(*E*-iminoether)$_2$(AcMet)$_2$ and the back reaction pseudo-first-order in *trans*-Pt(*E*-iminoether)$_2$(AcMet)(GMP). Under these conditions the plot of $\ln\{([\mathbf{H}]_0 - [\mathbf{H}]_\infty)/([\mathbf{H}] - [\mathbf{H}]_\infty)\}$ against time should give a straight line whose slope gives $k_1[GMP] + k_{-1}[AcMet]$. Knowing the ratio $k_1[GMP]/k_{-1}[AcMet] = [\mathbf{J}]_\infty/[\mathbf{H}]_\infty$ and the nearly constant values of free [GMP] and free [AcMet] it is possible to evaluate the second-order rate constants k_1 and k_{-1} (k_1 = 0.12 ± 0.02 M^{-1} s^{-1} and k_{-1} = 0.031 ± 0.006 M^{-1} s^{-1}). The lower value of k_{-1} with

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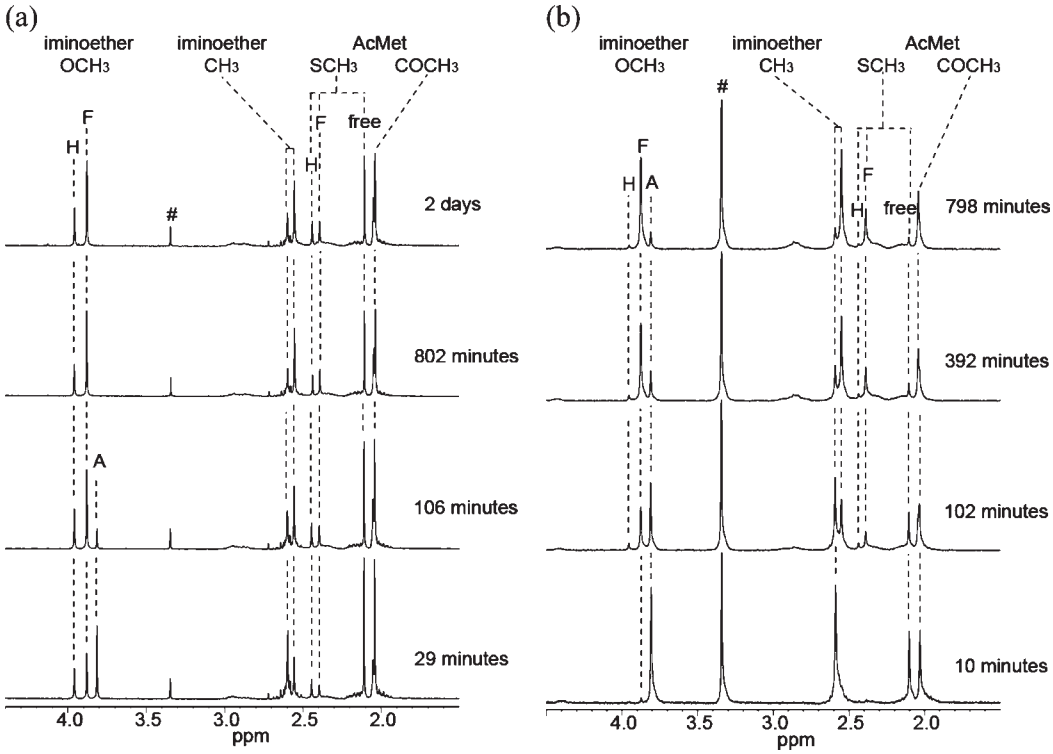

Figure 3. Reactions between *trans-EE* and AcMet performed at 25 °C and in the presence of 20 mM $NaClO_4$ and 100 mM NaCl: (a) [*trans-EE*]:[AcMet] = 1:2.5, pH 2.8 and (b) [*trans-EE*]:[AcMet] = 1:1, pH 3.1. Reaction time is given on each spectrum. The resonance assignment is given on the top of the spectra. The letters on the spectra indicate the species of Scheme 2 originating the signal. The symbol # denotes the CH_3OD added as reference.

respect to k_1 indicates that the ternary complex **J** is thermodynamically more stable than the bis-methionine complex **H**.

GMP Bisadduct Does Not React with AcMet. Unlike the reaction of **H** with GMP to give **J** and AcMet, which is an equilibrium with the equilibrium shifted toward **J** (Scheme 3), **J** reacts slowly with GMP to give the bis-GMP derivative **E** which, however, does not react with AcMet to give back **J** (no reaction after 2 weeks time). Therefore, the following order of stability can be deduced: *trans*-Pt(*E*-iminoether)$_2$(GMP)$_2$ (**E**) > *trans*-Pt(*E*-iminoether)$_2$(AcMet)(GMP) (**J**) > *trans*-Pt(*E*-iminoether)$_2$(AcMet)$_2$ (**H**). This observation confirms that the product of N coordination (GMP) is generally thermodynamically more stable than that of S coordination (AcMet) and is consistent with previous reports concerning analogous reactions performed on [Pt(dien)Cl]Cl[35,36] and *cis*-DDP.[24]

Isomerization and Chelate-Ring Closure of *trans-EE*/Met Adducts. The bisadduct **H** is stable in acidic condition. However, when the pH is adjusted to 7.0, a transformation takes place. The transformation has been characterized by ^{13}C, ^{15}N, and ^{195}Pt NMR spectroscopy using isotopically labeled Met. The assignment of ^{15}N,^{13}C-labeled Met resonances in the absence and presence of *trans-EE* was carried out using 2D ^1H,^{15}N and ^1H,^{13}C HSQC, ^1H,^{13}C HSQC-TOCSY, and ^1H,^{13}C HMBC spectra.

The bis-methionine adduct *trans*-Pt(*E*-iminoether)$_2$(Met-S)$_2$ (**H**) was generated by reaction of *trans-EE* with ^{15}N,^{13}C-labeled Met at pH 2.5. Binding to *trans-EE* of Met sulfur causes a large downfield shift of Met $C^{\varepsilon}H_3$ and $C^{\gamma}H_2$ signals, while it does not affect much the $C^{\beta}H_2$ and $C^{\alpha}H$ signals. The ^1H,^{195}Pt HSQC spectrum exhibits a $^{195}Pt/^1H$ cross-peak at −3312/2.4 ppm (Figure 5a) stemming from ^3J coupling between ^{195}Pt and Met $C^{\varepsilon}H_3$. The ^{195}Pt chemical shift at −3312 ppm confirms the [N_2S_2] coordination of **H**.[37,38] The minor cross-peak at −2860/2.3 ppm is assigned to the monoadduct *trans*-PtCl(*E*-iminoether)$_2$(Met-S) (**F**).[37,38]

When the solution was adjusted to pH 7.0, the ^{195}Pt NMR showed an instantaneous change with formation of a species at −3040 ppm. The ^{195}Pt chemical shift suggests a PtN_3S environment,[37,38] probably stemming from S to N head-to-tail rearrangement of one methionine. Indeed, the ^1H,^{195}Pt HSQC spectrum exhibits two $^{195}Pt/^1H$ cross-peaks at −3040/4.9 and −3040/2.2 ppm, corresponding to ^2J coupling between ^{195}Pt and NH_2 of one Met and ^3J coupling between ^{195}Pt and $C^{\varepsilon}H_3$ of a second Met (Figure 5b). The complete resonance assignment of both methionines was carried out with the aid of ^1H,^{13}C HSQC-TOCSY and ^1H,^{13}C HMBC spectra. The N-bound Met is characterized by a remarkable upfield shift of the $C^{\alpha}H$ proton signal, a small upfield shift of the $C^{\beta}H_2$, and negligible shifts of $C^{\gamma}H_2$ and $C^{\varepsilon}H_3$. Furthermore, the ^{15}N chemical shift of the N-bound Met ($^{15}N/^1H$ cross-peak at −15/4.9 ppm in the ^1H,^{15}N HSQC spectrum, Figure S3a, Supporting Information) indicates that the nitrogen is trans to a sulfur.[37] This compound still has a S-bound methionine; thus, it should correspond to *trans*-Pt(*E*-iminoether)$_2$(Met-S)(Met-N) (**I**). A S to N head-to-tail rearrangement of Met has already been proposed to occur in the Pt(dien)(Met) complex (dien = diethylenetriamine) at basic pH;[39] however, in this latter case the rate of isomerization appears to be much slower.

For longer reaction time at least two other products (**K** and **L**) were formed having ^{195}Pt at −3700 and −3640 ppm and $^{195}Pt/^1H$ correlation peaks with Met $C^{\varepsilon}H_3$ at 2.55 and 2.50 ppm, respectively (Figure 5c). We believe that these new compounds

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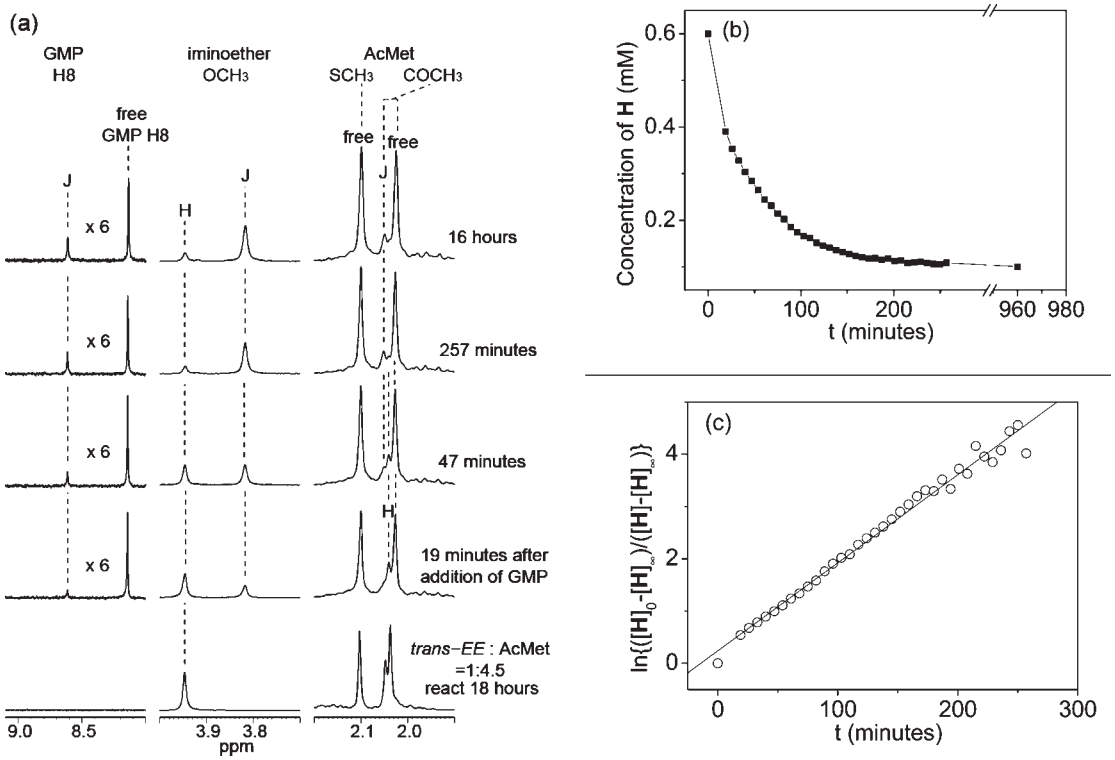


Figure 4. Reaction of 0.6 mM *trans*-Pt(*E*-iminoether)$_2$(AcMet)$_2$ with 2.0 mM GMP and 1.5 mM AcMet performed at pH 3.5 and at 25 °C. The resonance assignment is given on the top of the spectra; the letters on the spectra indicate the species of Scheme 3 originating the signal: (a) ^{1}H NMR spectra at various reaction times; (b) decrease in intensity of the OCH$_3$ peak of *trans*-Pt(*E*-iminoether)$_2$(AcMet)$_2$ (**H**) with reaction time; (c) plot of $\ln\{([\mathbf{H}]_0 - [\mathbf{H}]_\infty)/([\mathbf{H}] - [\mathbf{H}]_\infty)\}$ against reaction time.

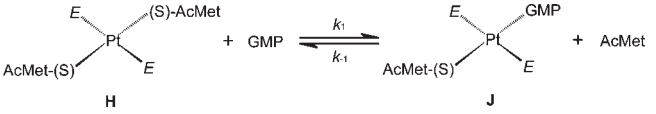
have the same composition and correspond to *cis*-Pt(Met-N,S)$_2$.[40] The ESI-MS on a sample at long reaction time confirmed formation of the Pt(Met-N,S)$_2$ complex (Figure S4, Supporting Information). The cis configuration is deduced from the chemical shifts of Met ^{15}N atoms (^{15}N/^{1}H cross-peaks at δ^{15}N = −20 and −19 ppm in the ^{1}H,^{15}N HSQC spectrum, Figure S3b, Supporting Information) whose values are indicative of nitrogens trans to sulfur atoms.[37,38] Formation of more than one product is due to different configurations (*S* or *R*) at the sulfur atoms (the configuration at sulfur is particularly stable when the methionine is chelated). In principle, three different diastereoisomers are possible (*RR*, *RS*, and *SS* configurations at the two sulfur atoms); however, there can be overlap of some resonances.[40] Simultaneously with formation of **K** and **L**, we also observed release of the iminoether ligands.

It is possible that between **I** and **K**/**L** there is formation of an intermediate species having a single chelated methionine, that is, *cis*-Pt(*E*-iminoether)(Met-S,N)(Met-S). This would be the first product of rearrangement of compound **I** having a N-bound Met with a free S eager to bind to platinum. The overall transformation **H** is undergoing at neutral pH is shown in Scheme 4.

DISCUSSION

Reaction with Excess Methionine. The reaction with methionine is fundamentally different for cis- and trans-oriented platinum drugs. In the case of *cis*-DDP, the first methionine replaces one chlorido ligand to form a 1:1 coordination product, *cis*-PtCl(NH$_3$)$_2$(Met-S).[25,41,42] Because of the strong trans-labilizing effect of sulfur, a second molecule of methionine can

Scheme 3. Equilibrium Reaction between *trans*-Pt(*E*-iminoether)$_2$(AcMet)$_2$ (H) and GMP



displace one ammine ligand forming *trans*-PtCl(NH$_3$)(Met-S)$_2$.[42] Subsequently, chelation of both methionines is observed with formation of the bis-chelated compound Pt(Met-S,N)$_2$.[24,25,42] Surprisingly, in the final product the two coordinated sulfur atoms are cis to one another (*cis*-Pt(Met-N,S)$_2$), although the entering of the second methionine is assumed to take place in the trans position (*trans*-PtCl(NH$_3$)(Met-S)$_2$).[42]

Also, in the case of trans compounds, such as *trans-EE*, the first step is substitution of one chloride and formation of the monoadduct. However, in this case the sulfur atom goes trans to a chloride (*trans*-PtCl(*E*-iminoether)$_2$(Met-S), **F**) and is this second chloride to be displaced by a second molecule of methionine, producing the bisadduct (*trans*-Pt(*E*-iminoether)$_2$-(Met-S)$_2$, **H**). In this investigation we discovered that this second substitution is very fast and, as a consequence, the bisadduct (**H**) dominates over the monoadduct (**F**). Formation of the bisadduct (**H**) is suppressed in the presence of high chloride concentration. Furthermore, complex **H** can be converted to the monoadduct **F** in the presence of excess *trans-EE* or of excess chloride ion. This observation indicates that *trans-EE* may interact with sulfur ligands already in the extracellular medium. In the presence of

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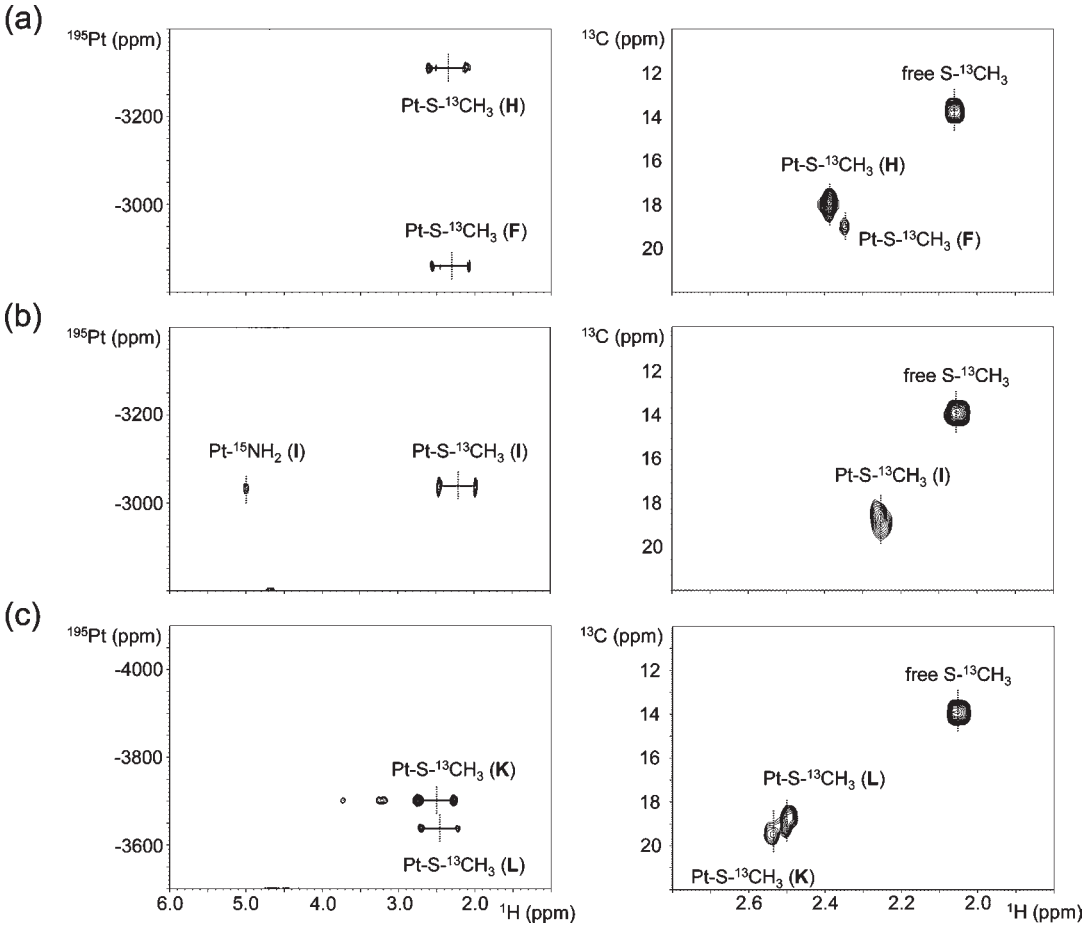
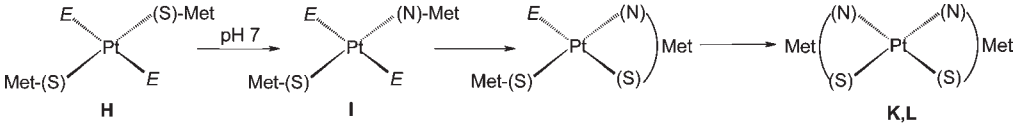


Figure 5. 2D ^{1}H,^{195}Pt HSQC (left, without ^{13}C decoupling, solvent H_2O) and ^{1}H,^{13}C HSQC (right, solvent D_2O) spectra of the reaction mixture of *trans-EE* and ^{15}N,^{13}C Met ([*trans-EE*]:[Met] = 1:2.5): (a) pH = 2.5, (b) after 30 min at pH = 7.0, and (c) after 48 h at pH = 7.0.

Scheme 4. Overall Transformation of H at pH 7.0



100 mM NaCl the monoadduct is by far the dominant species, while at low chloride concentration the bisadduct is favored. Therefore, a different reactivity can be envisaged for *trans-EE* in extra- and intracellular environments. *trans-EE* would form predominantly monoadducts in extracellular medium and bisadducts in the intracellular environment. The second-order rate constant for formation of the mono-methionine adduct in the presence of high chloride concentration (100 mM) was found to be 0.26 ± 0.01 M^{-1} s^{-1}. This means that in the presence of a thioether concentration of 1 mM the half-life for *trans-EE* is only 50 min. It is also to be noted that the rate of entering of a second thioether molecule is even faster as shown by the dominance of the bisadduct in the early stage of the reaction in the absence of excess chloride.

Isomerization of the Bis-Methionine Adduct and Chelation of Methionine. The sulfur-bound bis-methionine adduct **H** is stable for weeks in acidic condition; however, a rapid S to N isomerization of one methionine (compound **I**, NMR data) takes place in neutral conditions. The rapid head-to-tail rearrangement of one Met as the pH is raised from 2.5 to 7 can be explained by (a) deprotonation of the free aminic group of the S-bound Met, (b) the close proximity of the free aminic group to Pt, and (c) the greater stability of a *trans*-N−Pt−S as compared to a *trans*-S−Pt−S configuration (no competition between the two trans ligands for back-donation from Pt d orbitals). It is worth noting that, starting from **I**, chelation of the two methionines with displacement of the iminoether ligands would lead directly to formation of *cis*-Pt(Met-N,S)$_2$ having mutually cis sulfur atoms (shown in Scheme 4). The identity of this end product was confirmed by NMR.

The same final product (*cis*-Pt(Met-N,S)$_2$) is also obtained in the reaction of *cis*-DDP with excess methionine.[42] Also, in this case the intermediate bisadduct with monodentate methionines, *trans*-Pt(NH$_3$)Cl(Met-S)$_2$, has a trans geometry; therefore, isomerization to *cis*-Pt(NH$_3$)Cl(Met-S)$_2$ before chelation of Met has been hypothesized.[36] Such an isomerization would imply

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substitution of the Cl ligand by a third Met molecule with formation of $Pt(NH_3)(Met\text{-}S)_3$ followed by reentering of Cl^- to afford *cis*-$Pt(NH_3)Cl(Met\text{-}S)_2$. However, formation of *cis*-$Pt(Met\text{-}N,S)_2$ has been observed even in the absence of additional Met.[42] In light of our results, formation of the *cis*-$Pt(Met\text{-}N,S)_2$ end product can be easily explained by assuming a head-to-tail isomerization of one Met in *trans*-$Pt(NH_3)Cl(Met\text{-}S)_2$ to give *trans*-$Pt(NH_3)Cl(Met\text{-}S)(Met\text{-}N)$ (analogous to compound **I** formed in our case) followed by chelation of the N-bound Met to give *cis*-$Pt(NH_3)(Met\text{-}S)(Met\text{-}S,N)$ (the sulfur free end of the N-coordinated Met is a good entering group). After this step, chelation of the S-bound Met (favored by the trans-labilizing effect of the sulfur atom of the first chelated methionine) would take place. In conclusion, the head-to-tail rearrangement of a S-bound Met we discovered could be more general and apply also to other similar cases reported in the literature. It should be mentioned that this is not the first time that a head-to-tail rearrangement of S-bound Met has been proposed.[39]

Synergism between Met and GMP for Coordination to Platinum. The seminal work describing the reaction of the monofunctional platinum compound [Pt(dien)Cl]Cl with thioethers and GMP has provided clear evidence that thioethers can coordinate faster but can be displaced by guanine N-7, suggesting that sulfur coordination is kinetically preferred but nitrogen coordination is thermodynamically more stable and that sulfur compounds can deliver platinum to target DNA.[35,36] However, in the previous example (the thioether group trans to an aminic nitrogen) the rate of displacement of methionine by guanine N-7 is very slow,[36] much slower than that of direct reaction between *cis*-DDP and DNA.[36] Moreover, not only a methionine trans to a N-donor is displaced with difficulty by a guanine base, but the sulfur atom can also labilize the trans aminic ligand. This is the case of the *cis*-DDP/Met complex $Pt(Met\text{-}S,N)(NH_3)_2$ which reacts with GMP releasing one ammine group and forming $Pt(Met\text{-}S,N)(NH_3)(GMP)$.[24] The reaction is slightly faster than the direct reaction between cisplatin and GMP[24] but in the final compound is missing one ammine carrier ligand, while the presence of both ammines is retained essential for antitumor activity. Therefore, compounds like *cis*-$PtCl(Met\text{-}S)(NH_3)_2$ or *cis*-$Pt(Met\text{-}S,N)(NH_3)_2$ can provide alternative pathways for GMP binding to cisplatin, but the adduct could be inactive because of loss of one ammine.

Unlike *cis*-DDP, reaction of *trans-EE* with Met leads to *trans*-$PtCl(E\text{-iminoether})_2(Met\text{-}S)$ still containing a leaving chloride trans to sulfur. Moreover, the coordinated methionine can significantly accelerate reaction of substitution of chloride by GMP or DNA forming a ternary complex.[30] This result suggests that protein binding may facilitate DNA platination of trans-geometry platinum complexes, in striking contrast with traditional cis-geometry compounds.

Furthermore, even the bisadduct *trans*-$Pt(E\text{-iminoether})_2(AcMet)_2$ (**H**), which has already lost both leaving chlorides, can react readily with GMP affording the ternary complex *trans*-$Pt(E\text{-iminoether})_2(AcMet)(GMP)$. The second-order rate constant for displacement of one AcMet by GMP ($0.12 \pm 0.01\ M^{-1}\ s^{-1}$) is only one-half that of entering of the first AcMet on *trans-EE*. Moreover, the reaction is of equilibrium (AcMet can displace GMP in *trans*-$Pt(iminoether)_2(AcMet)(GMP)$, but the back reaction is ca. 4 times slower than the forward reaction). Therefore, also the bis-methionine derivative can represent a mean for platinum delivery to DNA.

Two Alternative Pathways for Formation of Coordinatively Bound DNA/Pt/Protein Ternary Complexes. It has been reported that ternary complexes protein/*trans-EE*/DNA can play an important role in inhibiting DNA repair and therefore draw cells to death by apoptosis.[29] Formation of protein/Pt/DNA ternary complexes can occur through two distinct pathways, either by reaction of the protein with a platinum−DNA complex or by reaction of DNA with a protein−platinum complex. Only the former pathway has received general consideration;[43,44] however, the present investigation indicates that formation of ternary complexes can take place also through the latter pathway. After administration, platinum drugs encounter several proteins, peptides, and other biomolecules before reaching the nuclear DNA.[10] The possibility that protein-bound platinum coordinates to DNA thus represents a realistic pathway in the mode of action of *trans*-platinum compounds. From the above discussion also emerges another profound difference between cis- and trans-oriented platinum complexes. The latter compounds can form readily coordinatively bound protein/Pt/DNA ternary complexes which could represent effective stop sites for DNA polymerase.[29] In contrast, *cis*-DDP is believed to form essentially binary complexes with target DNA and is the noncovalent interaction of the cisplatin-modified DNA with some proteins (such as HMG-B1) the key feature resulting in inhibition of RNA polymerase and of DNA repair.[43]

CONCLUSIONS

A detailed investigation has shown that in the reaction of *trans-EE* with methionine at low NaCl the bisadduct is formed rapidly and is the dominant species as long as free Met is present. In contrast, at high NaCl concentration the monoadduct is formed preferentially. These results suggest that different reaction products can be formed in the extra- or intracellular environment.

The bisadduct *trans*-$Pt(E\text{-iminoether})_2(AcMet)_2$, although completely deprived of leaving chlorido ligands, can react rather fast with GMP forming the coordinatively bound ternary adduct AcMet/*trans-EE*/GMP similar to that formed by the monoadduct *trans*-$Pt(E\text{-iminoether})_2(AcMet)Cl$. Altogether, these results indicate that coordination to DNA of platinum−protein adducts is highly feasible for trans-configuration platinum complexes.

It has also been shown that *trans*-$Pt(E\text{-iminoether})_2(Met)_2$ is stable in acidic condition, whereas an intramolecular S to N isomerization of one Met occurs at neutral pH. Such a head-to-tail rearrangement of a S-bound Met could be quite general and apply also to other cases reported in the literature, such as formation of *cis*-$Pt(Met\text{-}N,S)_2$ in the reaction of *cis*-DDP with excess methionine. The intermediate species, *trans*-$Pt(NH_3)Cl(Met\text{-}S)_2$, would not require isomerization to *cis*-$Pt(NH_3)Cl(Met\text{-}S)_2$ in order to give the final product if one Met just undergoes an intramolecular S to N isomerization.

ASSOCIATED CONTENT

Supporting Information. Kinetic plots, reaction pathways, NMR spectra and ESI-MS spectrum. This material is available free of charge via the Internet at http://pubs.acs.org.

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