A Novel Dinuclear Diaminoplatinum(II) Glutathione Macrochelate**

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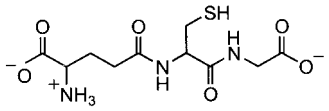
How do platinum anticancer drugs reach their DNA target sites in cells despite the presence of millimolar concentrations of the thiol tripeptide glutathione[1] (γ-L-Glu-L-Cys-Gly, GSH)? This is a long-standing puzzle, for which there is



GSH

currently no answer.[2] Reactions of platinum drugs with GSH in cells[3] are thought to be deactivating. Cisplatin-resistant tumor cells often contain elevated GSH levels, and transport of Pt out of cells is facilitated by GS-X pumps.[4] However, coadministration of cisplatin with GSH as a chemoprotectant does not appear to diminish the activity of cisplatin.[5] The major product from reaction of cysteine derivatives with *cis*-diam(m)inoplatinum(II) complexes is thought to be the bridged dimer $[(am)_2Pt(\mu_2\text{-}SR)_2Pt(am)_2]$, where am is am(m)ine or one-half of a diamine.[6–8] These bridged complexes are unlikely to be reactive towards DNA bases, although the ammine complexes can slowly release ammonia. There is now much interest in other types of adducts of GSH with *cis*-diamine complexes,[6, 9] which might be more reactive.

In this work we have isolated and characterized a new product formed by reactions of either GSH or glutathione disulfide (GSSG) with the anticancer complex $[Pt(en)Cl_2]$ (**1**, en = ethylenediamine) under physiologically relevant conditions: an unusual glutathione-bridged diplatinum(II) macrochelate. Although stable, it is potentially reactive, and such species could play an important role in the mechanism of action of platinum anticancer complexes.

We first studied reactions between **1** (100 μM) and GSSG or GSH (1:2 molar ratio, pH 7, 310 K) by HPLC. Representative chromatograms for the GSSG reaction are given in the Supporting Information (Figure S1, UV detection). Five new peaks appeared within the first few hours. One peak (C) was an intermediate, and after 24 h two peaks (D and E) predominated. The time course for this reaction, as determined by on-line HPLC-ICP-MS measurements of Pt concentrations, is shown in Figure 1b. Chromatograms for the GSH reaction were similar, although the reaction was slightly faster than that with GSSG (Figure 1; $t_{1/2} = 0.9$ h (GSH) and 1.3 h (GSSG)) and the ratio E:D was higher for the GSSG reaction.

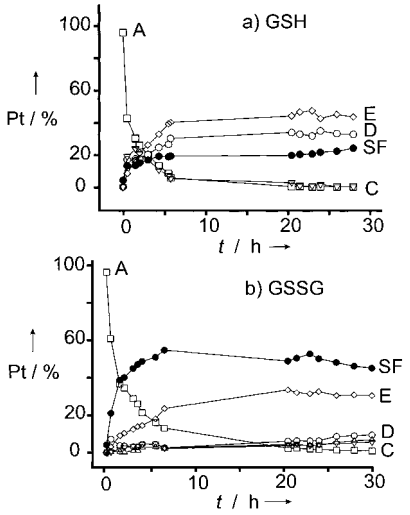
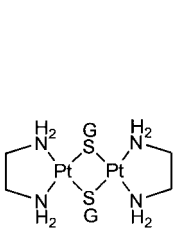


Figure 1. Time courses of the reactions of $[Pt(en)Cl_2]$ (**1**) with a) GSH and b) GSSG, as determined by measurements of Pt concentrations using HPLC-ICP-MS. Peak assignments: A: **1**; C: unknown; D: **2**; E: **3**; SF = solvent front (contains Pt species not retained by the column; for GSSG includes a Pt–GSSG adduct). See also Figure S1 in the Supporting Information.

The HPLC-ESI-MS experiments on reaction mixtures after 24 h showed that the two major species corresponding to peaks D and E were identical for GSSG and GSH reactions. Both gave a cluster of ions related to the isotopic distribution of Pt and N, with the species for peak D having its most abundant peak $[M^+]$ at m/z 1121 corresponding to $Pt_2S_2C_{24}N_{10}O_{12}H_{48}$ (calcd 1122; complex **2**), and that for peak E $[M^+ + H]$ at m/z 814 corresponding to $Pt_2SC_{14}N_7O_6H_{30}$ (calcd 814; complex **3**). A peak in the solvent front at m/z 866, corresponding to the isotopic distribution characteristic of $PtS_2C_{22}N_8O_{12}H_{40}$ ([Pt(en)(GSSG)]), was detected for the GSSG but not the GSH reaction.



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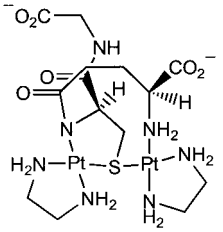

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The courses of the reactions of **1** with GSSG and GSH were also followed by 2D [^{1}H,^{15}N] HSQC NMR spectroscopy using [^{15}N]-**1**. After 24 h both reactions gave rise to the same set of nine major 2D NMR ^{1}H/^{15}N cross-peaks. These cross-peaks corresponded to the summation of peaks obtained from the

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separated HPLC peaks D and E. Peak D gave rise to a single $^{1}H/^{15}N$ cross-peak at $\delta = 5.1/-10.0$, characteristic of $Pt{-}^{15}NH_2$ *trans* to S.[10] When linked with the ESI-MS data, this suggests that peak D corresponds to the symmetrical S-bridged dimer $[\{Pt(en)(\mu_2\text{-}SG)\}_2]$ (**2**). Peak E gave rise to eight $^{1}H/^{15}N$ NMR cross-peaks (Table 1), consistent with the presence of two $\{Pt([^{15}N]\text{-}en)\}^{2+}$ units with two nonequivalent protons on each of the four nonequivalent ^{15}N atoms. The ^{15}N shifts suggest that two ^{15}N atoms are *trans* to S and two are *trans* to N/Cl. The ESI-MS data for peak E corresponded to a di-Pt(en) complex containing only one glutathione ligand. The structure of complex **3** was deduced from an extensive series of single-pulse 1D ^{1}H, $^{1}H\{^{15}N\}$, and 1D-TOCSY NMR experiments, combined with 2D homonuclear $^{1}H\text{-}^{1}H\{^{15}N\}$-DQFCOSY, TOCSY, ROESY, NOESY, and heteronuclear $[^{1}H,^{15}N]$ HSQC, HSQC-TOCSY (Figure 2), and HSQC-NOESY NMR experiments. The important results are summarized below and in Table 1.

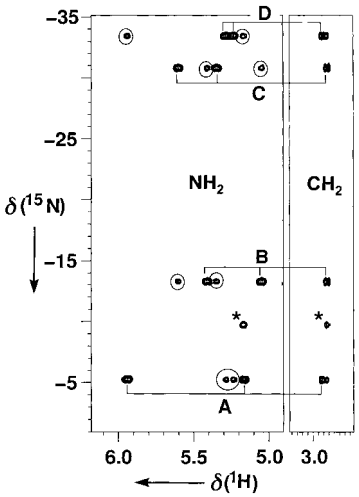

Figure 2. 2D $[^{1}H, ^{15}N]$ HSQC-TOCSY NMR spectrum (11.74 T, mixing time 54 ms) of the species eluted with chromatographic peak E (complex **3**). Four $H_2{}^{15}N\text{-}CH_2$ spin systems can be seen (A, B, C, D). Those belonging to the same $[^{15}N]$-en ligands are apparent from the circled cross-peaks (A/D, B/C). Resonances for the species from residual chromatographic peak D (complex **2**) are indicated by *.

Specific assignments were made for all of the ^{1}H and ^{15}N resonances of each $\{Pt([^{15}N]\text{-}en)\}^{2+}$ residue of **3**. For the coordinated glutathione, the Gly-N*H* resonance showed couplings to the doublet of doublets at $\delta = 4.18$ (Gly-Hα^{a}) and 3.61 (Gly-Hα^{b}). The doublet at $\delta = 5.05$ for Cys-Hα was coupled to the Cys-Hβ^{b} proton at $\delta = 2.76$, but not to its geminal partner Cys-Hβ^{a} at $\delta = 2.70$. No signal assignable to Cys-N*H* was observed. A spin system consisting of five protons in the region $\delta = 2.0-3.5$ was assigned to the $CHCH_2CH_2$ moiety of γ-Glu, and broadened resonances at $\delta = 6.20$ and 5.13 were assigned to γ-Glu-N*H*(1) and γ-Glu-N*H*(2), respectively. The former was a doublet consistent with a γ-Glu-NH(1) to γ-Glu-Hα torsion angle of about 90°. The latter resonance was a triplet with two large couplings, consistent with a torsion angle γ-Glu-NH(2) to γ-Glu-Hα of 180°. Broadening at the base of these resonances can be attributed to the presence of ^{195}Pt satellites (two-bond $^{195}Pt, ^{1}H$ coupling (^{2}J)) broadened by chemical shift anisotropy relaxation. Specific rOes were observed as follows: Gly-NH to en-NH(A), Gly-NH to Cys-Hα, Gly-NH to both Gly-Hα protons, γ-Glu-NH(1) to γ-Glu-Hα, γ-Glu-NH(2) to γ-Glu-Hα, γ-Glu-NH(1) to γ-Glu-Hγ^{a}, γ-Glu-Hγ^{a} to en-NH(A′). A particularly strong rOe was also observed between Cys-Hβ^{b} and γ-Glu-Hα. When a sample corresponding to chromatographic peak E was freeze-dried and redissolved in D_2O, all of the resonances assigned to NH protons disappeared except for that of γ-Glu-NH(1). This was now a singlet and subsequently disappeared slowly over a period of 24 h, suggesting a relatively buried environment.[11]

Figure 3 shows a model for complex **3** which is consistent with all these observations. The rOe between Gly-NH and

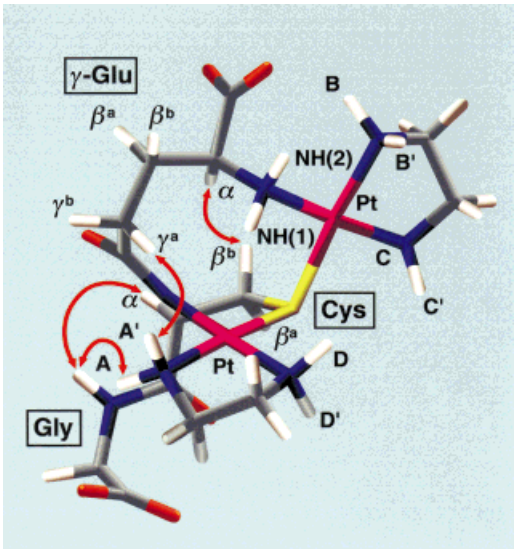

Figure 3. Structure for complex **3** (chromatographic peak E) which is consistent with observed NMR rOe (inter-residue rOes are indicated by arrows) and coupling constant data. Assignments for peaks B/B′, C/C′, and D/D′ are nonstereospecific.

Table 1. ^{1}H and ^{15}N NMR resonance assignments for complex $[^{15}N]$-**3** (chromatographic peak E).

	$^{15}NH_2$		CH_2		$^{15}NH_2$	
	$\delta(^{15}N)$ (*trans* to)	$\delta(^{1}H)$	$\delta(^{1}H)$	$\delta(^{1}H)$	$\delta(^{15}N)$ (*trans* to)	$\delta(^{1}H)$
$\{Pt([^{15}N]\text{-}en)\}$	–5.50 (S)	5.95 (A), 5.18 (A′)	2.84	2.70	–33.00 (N)	5.30 (D), 5.24 (D′)
	–13.37 (S)	5.41 (B), 5.05 (B′)	2.75	2.74	–30.50 (N)	5.60 (C), 5.35 (C′)
$H_{-2}\text{-}SG$	δ(N*H*)	δ(Hα)	δ(Hβ^{a})	δ(Hβ^{b})	δ(Hγ^{a})	δ(Hγ^{b})
Gly	7.86	4.18, 3.61				
Cys	–	5.05	2.70[a]	2.76[b]		
γ-Glu	6.20 (NH1),[c] 5.13 (NH2)	3.20	2.00	2.35	3.25	2.55

[a] $^{3}J(H,H) = 0$ Hz, $^{2}J(H,H) = 14$ Hz. [b] $^{3}J(H,H) = 5.9$ Hz, $^{2}J(H,H) = 14$ Hz. [c] Exchanges only slowly with ^{2}H.

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en-NH_2(A) places Gly on one side of one of the Pt^{II} coordination planes, and the rOe between γ-Glu-Hγ^a and en-NH_2(A′) places γ-Glu on the opposite side of the same Pt^{II} coordination plane. The large rOe between γ-Glu-Hα and Cys-Hβ^b is consistent with the close proximity of these two atoms in the model. The structure therefore consists of two square-planar (en)Pt^{II} centers bridged by a μ_2-thiolate S atom. One (en)Pt^{II} unit forms a five-membered ring by binding to the deprotonated amide N atom of Cys, and the other is coordinated to the γ-Glu NH_2 group, giving rise to a ten-membered macrochelate ring.

Complex **3** appears to be a novel adduct with an unprecedented structure. It is intriguing that this is a product from both GSH and GSSG reactions. Platinum(II)-induced disulfide bond cleavage has been reported previously, although the reactions studied were quite slow.[12–15]

Our preliminary HPLC and NMR studies show that **3** (but not **2**) reacts with the nucleotide guanosine 5′-monophosphate at micromolar concentrations in aqueous solution at neutral pH. Several Pt complexes containing chelated diamines are currently on clinical trial, and the discovery of complex **3** may therefore have significant implications for understanding their mode of action.

Experimental Section

$[Pt(en)Cl_2]$ (**1**) and [^{15}N]-**1** were synthesized according to previously described procedures.[16] Reduced glutathione (GSH) was purchased from Acros, and oxidized glutathione (GSSG) from Sigma.

The NMR data sets were acquired using Varian ^{UNITY}*INOVA* 600 and Bruker DMX 500 NMR spectrometers equipped with *z*-field gradient triple resonance NMR probes and operating at ^{1}H resonance frequencies of 599.841 and 500.13 MHz, respectively. Data acquisition and processing conditions have been described previously.[17]

Analytical separations were carried out on a BioCAD SPRINT Workstation using a Nucleosil 100-5SA cation-exchange column (250 × 4.6 mm, 100 Å, 5 μm, Hichrom) with ammonium acetate buffer (2 mM). The time courses of reactions between **1** (100 μM, 2 mL) and GSSG or GSH (20 mM, 20 μL) at 1:2 molar ratios were carried out at 310 K and followed chromatographically by injection of aliquots of the mixture onto the HPLC column at various time intervals after mixing, with detection at 223 nm. The pH values of GSSG and GSH solutions were adjusted to 7 before mixing with **1**.

Positive-ion electrospray mass spectrometry (ESI-MS) was performed on a Platform II mass spectrometer (Micromass, Manchester, UK). The atmospheric pressure ionization (API)-ESI ion source was interfaced to an LC Waters 2690 HPLC system using the same column and procedure as above, with a splitting ratio of 1/5. The source temperature was 413 K, and the drying gas flow rate was 450 L h^{-1}. A potential of 2.8 kV was applied to the probe tip, and a cone voltage of 50 – 90 V over 300 – 2000 Da was used. The quadrupole was scanned at 100 amu s^{-1}. The mass accuracy of all measurements was within 0.1 *m*/*z* units.

An inductively coupled plasma mass spectrometer (ICP-MS) Plasma Quad 3 from VG Elemental (Winsford, UK) was used for Pt determinations. The ICP-MS was coupled with a Gilson 305 pump, a Gilson manometric module, and a Rheodyne sample injector through PEEK tubing connected directly to the Meinhardt concentric nebulizer. Data were acquired at mass 195, using the VG software for time-resolved analysis, with a dwell time of 300 ms. Chromatographic separations were carried out as described above at a flow rate of 0.8 mL min^{-1}, using a 20-μL sample loop. Samples were diluted to 1:1000 before injection to give concentrations of approximately 100 nM Pt.

The pH measurements were made using a Corning 240 pH meter equipped with an Aldrich microcombination electrode standardized with buffers at pH 4, 7, and 10.

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