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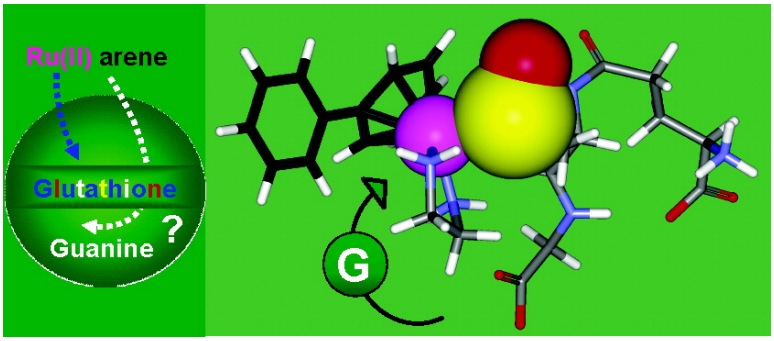

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Competition between Glutathione and Guanine for a Ruthenium(II) Arene Anticancer Complex: Detection of a Sulfenato Intermediate

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Abstract: The organometallic anticancer complex [(η^6-bip)Ru(en)Cl]$^+$ (**1**; bip = biphenyl, en = ethylenediamine) selectively binds to guanine (N7) bases of DNA (Novakova, O.; Chen, H.; Vrana, O.; Rodger, A.; Sadler, P. J.; Brabec, V. *Biochemistry* **2003**, *42*, 11544−11554). In this work, competition between the tripeptide glutathione (γ-L-Glu-L-Cys-Gly; GSH) and guanine (as guanosine 3′,5′-cyclic monophosphate, cGMP) for complex **1** was investigated using HPLC, LC−MS and ^{1}H,^{15}N NMR spectroscopy. In unbuffered solution (pH ca. 3), the reaction of **1** with GSH gave rise to three intermediates: an S-bound thiolato adduct [(η^6-bip)Ru(en)(GS-*S*)] (**4**) and two carboxylate-bound glutathione products [(η^6-bip)Ru(en)(GSH-*O*)]$^+$ (**5**, **6**) during the early stages (<6 h), followed by en displacement and formation of a tri-GS-bridged dinuclear Ru^{II} complex [((η^6-bip)Ru)$_2$(GS-μ-*S*)$_3$]$^{2-}$ (**7**). Under physiologically relevant conditions (micromolar Ru concentrations, pH 7, 22 mM NaCl, 310 K), the thiolato complex **4** was unexpectedly readily oxidized by dioxygen to the sulfenato complex [(η^6-bip)Ru(en)(GS(O)-*S*)] (**8**) instead of forming the dinuclear complex **7**. Under these conditions, competitive reaction of complex **1** with GSH and cGMP gave rise to the cGMP adduct [(η^6-bip)Ru(en)(cGMP-*N7*)]$^+$ (**10**) as the major product, accounting for ca. 62% of total Ru after 72 h, even in the presence of a 250-fold molar excess of GSH. The oxidation of coordinated glutathione in the thiolato complex **4** to the sulfenate in **8** appears to provide a facile route for displacement of S-bound glutathione by G N7. Redox reactions of cysteinyl adducts of these Ru^{II} arene anticancer complexes could therefore play a significant role in their biological activity.

Introduction

Several ruthenium(III) am(m)ine complexes such as *cis*-[RuCl$_2$(NH$_3$)$_4$]Cl and Na[*trans*-Ru(Im)$_2$Cl$_4$] exhibit anticancer activity, and are thought to be activated by reduction (to Ru^{II}) in vivo which facilitates DNA binding.[1] Recently we have shown that members of the family of Ru^{II} arene complexes [(η^6-arene)Ru(YZ)(X)][PF$_6$], where X is a halide and YZ is a chelating diamine, in which the presence of the arene greatly stabilizes Ru^{II} compared to Ru^{III},[2] are cytotoxic to cancer cells including cisplatin-resistant cell lines.[3] For chloro (X) ethylenediamine (YZ; en) complexes, the cytotoxicity increases with the size of coordinated arene in the order: benzene < *p*-cymene < biphenyl < dihydroanthrancene < tetrahydroanthrancene. The activity of several of the complexes against human ovarian cancer cell line A2780 is at least comparable to that of carboplatin, and some approach that of cisplatin. Activity has also been demonstrated against human xenographs in vivo.[3a]

DNA is a potential target for en Ru^{II} arene complexes, and they exhibit a high selectivity for binding to N7 of guanine.[4] DNA platination is thought to be a key event in the mechanism of action of platinum anticancer drugs such as cisplatin,[5] whereas the interaction of platinum species with sulfur-containing biomolecules has been associated both with negative phenomena (such as toxic side effects and the development of resistance) and with positive effects (such as delivery of active species to cells and/or serving as a drug reservoir for ultimate platination of DNA).[6] Platinum(II) has a high affinity for sulfur-containing biological molecules. The competitive reactions of N-donor (G^{N7}-DNA) and S-donor (thiol/thioether) ligands for Pt^{II} have been widely studied.[7] However, those between Ru^{II} arene anticancer complexes, N-donor nucleotides, and S-donor amino

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acids and peptides are largely unexplored, although we have demonstrated that both cysteine and methionine can form S-bound adducts with [(η^6-bip)Ru(en)Cl][PF_6] (**1**; bip = biphenyl).[8] Especially important is the tripeptide glutathione (γ-L-Glu-L-Cys-Gly; GSH), an abundant (millimolar) intracellular thiol responsible for the detoxification of heavier transition metal ions, including some platinum and ruthenium anticancer complexes.[1a,6]

The present work is focused on competitive reactions of the Ru^{II} anticancer complex [(η^6-bip)Ru(en)Cl][PF_6] (**1**) with GSH and guanosine-3′,5′-cyclic monophosphate (cGMP). Cyclic-GMP is a phosphate diester, as are the nucleotides in DNA and RNA. In particular, we have attempted to study reactions under physiologically relevant conditions: micromolar Ru concentrations, pH 7, and in the presence of a large molar excess of GSH. Surprisingly S-bound glutathione was found to be susceptible to oxidation, and reactions carried out in water without buffering followed a different course. Our findings suggest that novel redox reaction pathways could contribute to the biological activity of organometallic Ru^{II} arene complexes.

Experimental Section

Materials. [(η^6-bip)RuCl(en)][PF_6] (**1**) and ^{15}N-labeled **1** (^{15}N-**1**) were synthesized as described elsewhere.[3b,4a] Glutathione (GSH, reduced), guanosine 3′,5′-cyclic monophosphate (cGMP) sodium salt, disodium hydrogen phosphate, and Chelex resin (used for removal of impurity ions from phosphates) were purchased from Sigma; sodium dihydrogen phosphate and the ruthenium standard for atomic spectrometry (1003 μg Ru mL^{-1}) from Aldrich; sodium hydroxide and sodium chloride from Fisher; and trifluoroacetic acid (TFAH) from Acros.

High Performance Liquid Chromatography (HPLC). A Hewlett-Packard series 1100 quaternary pump and a Rheodyne sample injector with 100 μL and 2.0-mL loops, a HP 1100 series UV−vis detector and HP 1100 series Chemstation with a HP enhanced integrator were used. Analytical separations were carried out on a PLRP-S reversed-phase column (250 mm × 4.6 mm, 100 Å, 5 μm, Polymer Labs), and semipreparative work on a PLRP-S reversed-phase column (250 mm × 7.5 mm, 100 Å, 8 μm, Polymer Labs) with detection at 254 nm. Mobile phase A: water (for HPLC application, Fisher Chemicals) containing 0.1% TFAH; mobile phase B: acetonitrile (for HPLC application, Fisher Chemicals) containing 0.1% TFAH. For analytical assays, the flow rate was 1.0 mL min^{-1}, for semipreparative work, 3.5 mL min^{-1}. The gradient (solvent B) was as follows: 2% to 28% within 20 min, 80% from 21 to 24 min, reset to 2% from 26 to 30 min.

Electrospray Ionization Mass Spectrometry (ESI-MS). Positive-ion electrospray ionization mass spectra were obtained with a Platform II mass spectrometer (Micromass, Manchester, U.K.). A Waters 2690 HPLC system was interfaced with the mass spectrometer, using the same column and gradients as described above for the analytical HPLC separation, with a flow rate of 1.0 mL min^{-1} and a splitting ratio of 1/5. The spray voltage was 3.50−3.68 kV, and the cone voltage, 20 V. The capillary temperature was 410 K with a 450 L h^{-1} flow of nitrogen drying gas. The quadrupole analyzer, operated at a background pressure of 2 × 10^{-5} Torr, was scanned at 700−900 Da s^{-1}. Data were collected and analyzed on a Mass Lynx (ver. 3.5) Windows NT PC data system using the Max Ent Electrospray software algorithm and calibrated versus an NaI calibration file. The mass accuracy of all measurements was within 0.1 *m*/*z* unit.

Nuclear Magnetic Resonance (NMR) Spectroscopy. For HPLC-isolated glutathione products, NMR data were acquired at a temperature of 298 K using Bruker Avance 600 MHz NMR spectrometers equipped with a triple resonance TXI (^{1}H,^{13}C,^{15}N) *z*-gradient cryo-probe or room temperature TXI (^{1}H, ^{13}C, ^{15}N) triple-axis (*x*,*y*,*z*) gradient probehead.

One-dimensional (1D) ^{1}H NMR data were acquired with eight transients into 32k data points over a frequency width of 9.0 kHz using either water presaturation or a double-pulsed field gradient spin−echo routine (DPFGSE) to eliminate the solvent resonance.[9]

Two-dimensional (2D) [^{1}H,^{1}H] DQFCOSY, TOCSY, and NOESY NMR data were acquired over a frequency width of 5.4 kHz in both F_2 and F_1 into 2k complex data points in F_2 (acquisition time = 190 ms) with two transients for each of 2 × 512 t_1 increments in the QF (COSY) or the States - TPPI (TOCSY and NOESY) mode. A relaxation delay of 1.4 s between transients was used for all experiments. 2D NOESY NMR data were acquired with mixing times of 100 and 400 ms, and 2D TOCSY data with a spin-lock time of 60 ms. Water suppression for COSY was achieved using water presaturation. For TOCSY and NOESY experiments, the water resonance was suppressed by means of a DPFGSE routine after the final read pulse. Data were processed using standard apodizing functions prior to Fourier transformation.

The time courses of the reactions between ^{15}N-**1** and GSH and cGMP were followed by NMR using a Bruker Avance NMR spectrometer operating at 800 MHz. All data were acquired on samples equilibrated at 310 K. A triple resonance (TBI: ^{1}H,^{13}C,X) probehead equipped with a triple-axis (*x*,*y*,*z*) gradient coil was used for data accumulation. Calibration and spectrometer setup was carried out using a sample of ^{15}N-**1**, prior to the addition of GSH or/and cGMP.

1D ^{1}H NMR spectra were acquired without ^{15}N-decoupling over an 8 kHz frequency width for eight transients into 16k data points (acquisition time = 1.022 s) with a relaxation delay of 1.5 s between transients. ^{1}H pulse calibration was carried out using a presaturation pulse sequence (zgpr). Subsequently, ^{1}H NMR data were acquired either using a DPFGSE routine or its modified form in which composite inversion pulses were applied.[10] Gradients were used to select for ^{1}H−^{15}N coherences and were simultaneously responsible for eliminating the water resonance.

2D [^{1}H,^{15}N] HSQC NMR data were acquired, with ^{15}N-decoupling during the acquisition period, over an F_2 frequency width of 8 kHz (acquisition time = 128 ms). Multiples of eight transients were accumulated for each of 128 t_1 increments over an F_1 frequency width of 80 ppm centered at −30 ppm relative to $^{15}\underline{N}H_4Cl$ (reference, 0 ppm). Phase-sensitive data were acquired in a sensitivity-improved manner using an echo−antiecho acquisition mode.[11]

All NMR data were processed using Xwin-nmr (version 3.5, Bruker Biospin. Ltd.).

pH Measurements. pH measurements were made using a Corning 240 pH meter equipped with an Aldrich micro-combination electrode calibrated with Aldrich standard buffer solutions of pH 4, 7, and 10. For NMR samples prepared in 10% D_2O/90% H_2O, no correction has been applied for the effect of deuterium on the glass electrode.

Preparation of Samples. (a) HPLC. Reaction mixtures of complex **1** with GSH and/or cGMP at various molar ratios were prepared by mixing aliquots of 10 mM **1**, 50−500 mM GSH, and 20−100 mM cGMP. For the reactions under physiologically relevant conditions

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(phosphate buffer pH 7.0, 22 mM NaCl), the pH values of all starting solutions were adjusted to 7 using NaOH and $HClO_4$, and the O_2 content was minimized by bubbling with N_2 before and after mixing unless otherwise stated. The mixtures were diluted to the required concentration with deionized water or with 10−50 mM phosphate buffer solution (pH 7.0, purged with N_2), bubbled with N_2 again, and then incubated at 310 K in a water bath for the required times for the subsequent HPLC and LC−MS analysis or preparative separation. The samples for semipreparative separation contained 1−10 mM complex **1** and 5−50 mM GSH.

For the reaction of complex **1** with GSH under argon, the separate reactant solutions (pH of GSH solutions adjusted to 6.8) were mixed at molar ratios of 0.02:5 mM or 1:10 mM after deoxygenation by four cycles of freeze−thawing on a vacuum line under Ar, and then incubated at 310 K for 48 h under Ar bubbling. For the same reactions under O_2, similar solutions of the starting materials were mixed in air and then incubated at 310 K for 48 h with O_2 bubbling.

The time-courses of reactions of complex **1** (0.3 mM) with GSH (3 mM) in unbuffered aqueous solution in air (i.e. not purged with N_2), and of **1** (20 μM) with GSH (5 mM) and cGMP (0.5 mM) in phosphate buffer (pH 7) containing 22 mM NaCl under N_2 (all starting solutions purged by N_2 bubbling) at 310 K were followed chromatographically by injection of aliquots of the mixtures onto the HPLC column at various time intervals. Sampling at various times was done in air by briefly removing the cap to extract an aliquot with no further N_2 bubbling.

(b) NMR. The HPLC fractions from the reactions of complex **1** with various molar ratios of GSH in water or in phosphate buffer (pH 7) were collected from semipreparative HPLC separations, and immediately frozen in liquid nitrogen, and then lyophilized. The resulting solids were redissolved in 0.6 mL of 10% D_2O/90% H_2O for 1D and 2D ^1H NMR experiments. The pH value (initially pH ca. 2 due to the presence of trifluoroacetic acid) of the fraction containing complex **4** from the reaction of complex **1** with GSH in buffer solution (pH 7) was immediately adjusted to 7 using NaOH and $HClO_4$. The solution was then freeze-dried and redissolved in 90% H_2O/10% D_2O, and the pH was readjusted to 7 before the NMR experiments.

A 10 mM solution of ^{15}N-labeled complex **1** (^{15}N-**1**) was used to follow reactions of **1** with GSH and/or cGMP by 1D [^1H] and 2D [^1H,^{15}N] HSQC NMR. The reaction mixtures were prepared using the same method as that for HPLC samples except that for the reaction of **1** with GSH and cGMP, for which the O_2 content was minimized by bubbling with N_2 before and after mixing.

Determination of Extinction Coefficients. HPLC fractions of complex **1**, its aqua and TFA adducts as well as the GSH and cGMP adducts were collected from a 10 mM equilibrium aqueous solution of **1**, the 2-h and 24-h reaction mixtures of complex **1** with GSH in water, 24-h reaction mixture in buffer containing 22 mM NaCl, and 30-h reaction mixture of complex **1** with GSH and cGMP in buffer. After lyophilization, the residue from each fraction was dissolved in 5 mL deionized water for Ru determination by ICP-OES, using a Perkin-Elmer Optima 5300 DV Optical Emission Spectrometer equipped with an AS-93plus autosampler and WinLab32 for ICP program (version 3.0.0.0103). The apparent extinction coefficient at 254 nm (ϵ'_{254}) for each species was calculated using eq 1.

$$\epsilon'_{254} = [\text{peak area}]_{254}/[\text{Ru}]\ (\mu\text{mol}) \quad (1)$$

The relative extinction coefficient ϵ^{R}_{254} for each species was calculated from eq 2:

$$\epsilon^{R}_{254} = [\epsilon'_{254}]_x/[\epsilon'_{254}]_{\mathbf{1}} \quad (2)$$

where $[\epsilon'_{254}]_x$ and $[\epsilon'_{254}]_{\mathbf{1}}$ represent the extinction coefficients of species x and complex **1**, respectively, and the values are listed in Table 1.

Table 1. Relative Extinction Coefficients at 254 nm of HPLC-Isolated Ru Arene Complexes

complex	Ru[a] /μg mL^{-1}	Ru /nmol	peak area[b]	ϵ^{R}_{254}[c]
[(η^6-bip)Ru(en)Cl]$^+$ (**1**)	7.72	382	179358	1.00
[(η^6-bip)Ru(en)(H_2O)]$^{2+}$ (**2**)	6.30	312	161914	1.10
[(η^6-bip)Ru(en)(TFA)]$^+$ (**3**)	0.89	44.1	23722.9	1.14
[(η^6-bip)Ru(en)(GS-*S*)] (**4**)	0.74	36.8	36947.2	2.32
[(η^6-bip)Ru(en)(GS-*O*)]$^+$ (**5**)	1.06	52.6	29707.4	1.20
[(η^6-bip)Ru(en)(GS-*O*)]$^+$ ((**6**)	0.89	43.8	25517.4	1.24
[((η^6-bip)Ru)$_2$(GS-*S*)$_3$]$^{2-}$ (**7**)	0.97	47.8	42539.9	1.89
[(η^6-bip)Ru(en)(GS(O)-*S*)] (**8**)	0.41	20.3	11160.2	1.17
[(η^6-bip)Ru(en)(cGMP-*N7*)]$^+$ (**10**)	1.86	91.9	113369	2.62

[a] Ru concentration in 5 mL aqueous sample determined by ICP-OES. [b] Relative peak areas of HPLC fractions with UV detection at 254 nm. [c] Relative extinction coefficient.

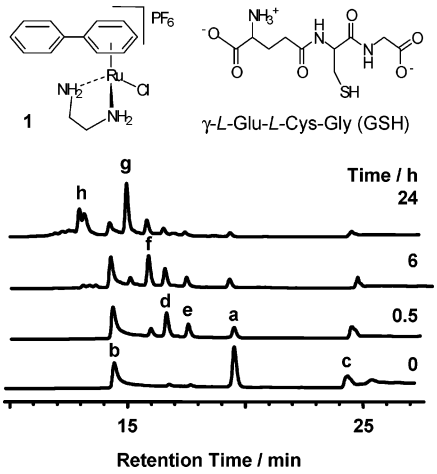


Figure 1. HPLC time-course for the reaction of **1** (0.3 mM) with 10 mol equiv GSH in aqueous solution (pH ca. 3) at 310 K. Peak assignments: (a) **1**; (b) [(η^6-bip)Ru(en)(H_2O)]$^{2+}$ (**2**); (c) [(η^6-bip)Ru(en)(TFA)]$^+$ (**3**); (d, e, f, g, and h) GSH adducts. Peak h appears to be due to tetranuclear clusters.

Results

Reaction of Complex 1 with GSH in Unbuffered Aqueous Solution. First, the reaction of complex **1** with GSH in water without adjustment of pH was investigated using HPLC, ESI-MS, and NMR spectroscopy. Reaction of **1** (0.3 mM) with a 10-fold molar excess of GSH (pH initially ca. 3) at 310 K gave rise to two products as detected by HPLC (peaks d, e in Figure 1) after ca. 10 min, and then another product (peak f) after 0.5 h. After 6 h, a forth adduct (peak g) formed and increased in concentration until 24 h; meanwhile HPLC peaks d, e, and f decreased in intensity. ESI-MS analysis of the HPLC fractions (Figure S1) gave a singly charged ion peak centered at *m*/*z* 622.1 for peaks d, e, and f, assignable to (isomers of) monoruthenium glutathione complexes **4**, **5**, and **6** (calcd *m*/*z* 622.1 for {(η^6-bip)Ru(en)(GS) + H}$^+$).[12] A fragment ion peak at *m*/*z* 561.9 resulting from release of en (calcd *m*/*z* 562.1 for {(η^6-bip)-Ru(GS) + H}$^+$) was detectable only for fraction f (adduct **4**).

The mass spectrum (Figure S1) of peak g indicated that this fraction contained a diruthenium glutathione adduct [((η^6-bip)-Ru)$_2$(GS)$_3$]$^{2-}$ (**7**) (calcd *m*/*z* 715.6 for {((η^6-bip)Ru)$_2$(GS)$_3$ + 4H}$^{2+}$). This fraction was also collected from a reaction mixture of complex **1** (5 mM) with GSH (25 mM) incubated at 310 K for 48 h and was characterized by 1D ^1H and 2D [^1H,^1H] NMR

(12) Under the conditions used in this work, coordinated GS has an overall charge of −2. Similarly coordinated GSO and GSO_2 are assumed to have charges of −2 in the formulae.

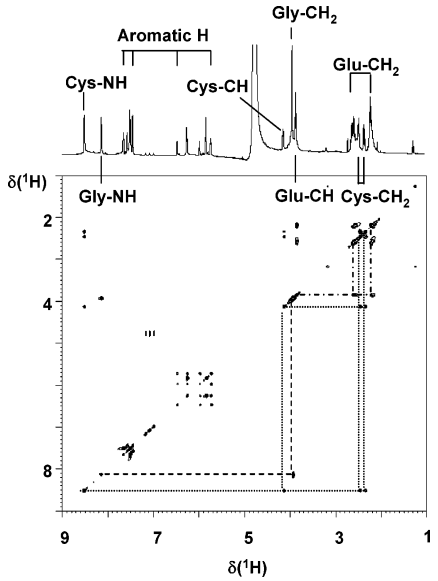


Figure 2. 1D ^{1}H and 2D [^{1}H,^{1}H] TOCSY NMR spectra for HPLC fraction (g) (see Figure 1) from the reaction of complex **1** with GSH (5:25 mM) in aqueous solution for 48 h at 310 K.

Table 2. ^{1}H NMR Chemical Shifts (δ) for GSH and GSH Ligands in the Adducts [(η^6-bip)Ru(en)(GS-*S*)] (**4**) and [((η^6-bip)Ru)$_2$(GS-μ-*S*)$_3$]$^{2-}$ (**7**) in 90% H_2O/10% D_2O (298 K)

	δ (^{1}H) ($\Delta\delta$)[a]			
	pH			
	GSH		**4**	**7**
proton	2.9	7.3	7.3	2.9
			Glu	
γ-CH_2	2.52	2.52	2.54 (0.02)	2.59 (0.07)
β-CH_2	2.13	2.13	2.20 (0.07)	2.19 (0.06)
α-CH	3.79	3.74	3.82 (0.08)	3.88 (0.09)
α-NH_3^+	—[b]	—[b]	—[b]	—[b]
			Cys	
β-CH_2	2.95	2.95	2.96 (0.01)	2.44 (−0.51)
	2.90	2.90	2.81 (−0.09)	2.32 (−0.58)
α-CH	4.54	4.54	4.38 (−0.16)	4.12 (−0.42)
α-NH	8.44	8.21	8.07 (−0.14)	8.51 (0.07)
			Gly	
α-CH_2	3.94	3.94	3.81 (−0.13)	3.93 (−0.01)
α-NH	8.48	8.25	8.31 (0.06)	8.16 (−0.32)

[a] $\Delta\delta = \delta$ (**4**, **7**) $- \delta$ (GSH). [b] Not observed (fast exchange).

spectroscopy (Figures 2, 3, and S2). Only one set of proton resonances was observed for the two biphenyl ligands (Figure S2, Table S1) and one set for the three glutathione ligands (Figure 2, Table 2). No peaks for en protons (en-NH_2 or en-CH_2) were detectable. For the biphenyl groups, five well-separated resonances are observed for the ortho (δ 7.46, 7.26), meta (δ 5.72, 5.83), and para (δ 5.97) protons of the coordinated phenyl ring (A), and four peaks for the ortho (δ 7.57), meta (δ 7.65, 7.51), and para (δ 7.45) protons of the noncoordinated phenyl ring (B). At pH 2.9, the β-CH_2 and α-CH proton resonances of the cysteine residues in adduct **7** are shifted to high field by 0.51, 0.58, and 0.42 ppm, respectively, compared with free GSH at the same pH value (Table 2). The 2D [^{1}H,^{1}H] NOESY NMR spectrum (Figure 3) shows that there are strong NOEs between the aromatic protons of the coordinated biphenyl ring (A) and the α-CH and β-CH_2 protons of the cysteine residues in **7**.

ICP-OES calibration of HPLC peak areas (Table 1) showed that after 2 h of the reaction 9% of the Ru was present as **4** and 31% as **5** + **6**. After 24 h, the dinuclear complex **7** was the main product (35%). In addition, during the late stages (>24 h), a doublet HPLC peak h was detectable (Figure 1). This is assignable to Ru clusters consisting of {(η^6-bip)Ru}$^{n+}$ units and GS^{2-}, GSO^{2-}, or GSO_2^{2-} ligands.[13] Peak h was also observed in the HPLC trace of the reaction mixture of **1** with 5 mol equiv of GSH after 24 h, but was not detectable for the reaction of **1** with 1 mol equiv of GSH under the same conditions (data not shown).

The reaction of complex **1** with GSH in unbuffered solution was also studied at millimolar concentrations (**1**:GSH 2:20 mM) at 310 K by 2D [^{1}H,^{15}N] HSQC NMR using ^{15}N-labeled **1** (^{15}N-**1**). The spectra are shown in Figure S3, and the ^{1}H and ^{15}N chemical shifts are listed in Table 3. The results are consistent with those obtained by HPLC. During the early stages (<1 h), three pairs of new cross-peaks appeared which are assignable to the ^{15}N-en ligands in adducts **4**, **5**, and **6**. Cross-peaks 5a/b and 6a/b (Figure S3) decreased in intensity after ca. 4 h and disappeared after ca. 10, and cross-peaks 4a/b increased in intensity up to 6 h, and then decreased and disappeared after 13 h. Because the diruthenium adduct **7** does not contain bound ^{15}N-en, it does not give rise to ^{1}H,^{15}N cross-peaks, and therefore no ^{1}H/^{15}N signals were detectable during the late stages (>13 h) of the reaction when adduct **7** was the main product.

Reaction of Complex 1 with GSH under Physiologically Relevant Conditions. The reaction of complex **1** at micromolar concentrations **(**20 μM) with 250 mol equiv of GSH was studied in phosphate buffer (10 mM) pH 7, containing 22 mM NaCl at 310 K.[14] To minimize the content of O_2, all starting solutions were purged of air by bubbling with N_2 before and after mixing.

The reaction course was different from that described above for unbuffered reactions. During the early stages (<12 h), HPLC peak i was dominant, but later (48 h) peak j increased in relative intensity (Figure 4). The mass spectrum of fraction i is identical to that of fraction f from the reaction of **1** with GSH in unbuffered aqueous solution (Figures S1 and S4), and corresponds to the thiolato adduct [(η^6-bip)Ru(en)(GS)] (**4**). For fraction j, a singly charged ion peak at *m*/*z* 638.3 was observed, corresponding unexpectedly to the sulfenato complex [(η^6-bip)-Ru(en)(GS(O))] (**8**) (calcd *m*/*z* 638.1 for {(η^6-bip)Ru(en)-(GS(O)) + H}$^+$), accompanied by a singly charged ion peak at *m*/*z* 324.0 corresponding to the release of the fragment {GSOH + 2H}$^+$ (calcd *m*/*z* 324.1; Figure S4). The amount of **8** in the reaction mixture increased from 11% (of the total Ru) after 12 h to 19% after 48 h.

From a reaction mixture containing complex **1** (1 mM) and GSH (25 mM) in phosphate buffer (50 mM, pH 7) and 22 mM NaCl which had been incubated at 310 K for 24 h, the thiolato adduct [(η^6-bip)Ru(en)(GS)] (**4**) was isolated by preparative HPLC and characterized by 1D and 2D ^{1}H NMR spectroscopy. The pH of the HPLC fraction was adjusted to 7, and after lyophilization the sample was dissolved in 90% H_2O/10% D_2O

(13) High-resolution FT-ICR-MS analysis (with S. Weidt and P. R. R. Langridge-Smith) showed doubly charged ion peaks centered at *m*/*z* 1006.0269, 1083.0602, 1172.0735, and 1180.5703, suggesting the formation of tetranuclear clusters.

(14) The concentrations of **1**, GSH, and NaCl were chosen so as to be within the ranges present in the cell cytoplasm.

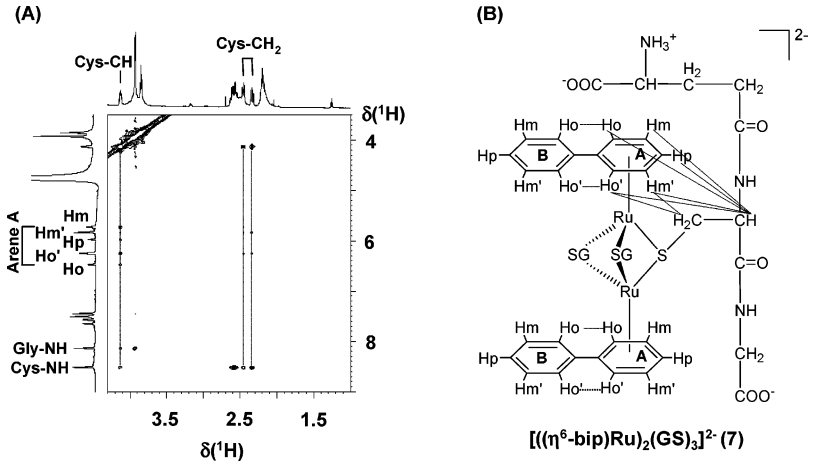


Figure 3. (A) NOEs between the protons of the biphenyl ligand and cysteine α-CH, β-CH_2 protons of **7**, indicating their proximity. (B) Structure of **7** with NOEs indicated by dotted lines; the side chains of two of the GS ligands are omitted for clarity. For NOEs between protons of the two phenyl rings, see Figure S2.

Table 3. ^1H,^{15}N NMR Peaks Observed for Reactions of ^{15}N-**1** (2 mM) with GSH (20 mM) in 90% H_2O/10% D_2O (pH ca. 2.9, Figure S3), and of ^{15}N-**1** (1 mM) with GSH (5 mM) and cGMP (1 mM) in 50 mM Phosphate Buffer (pH 7) and 22 mM NaCl (Figure S10) at 310 K

complex	(peak) δ $^1H/^{15}N$	
1	(1a) 6.09/−24.62	(1b) 4.05/−24.62
2 (pH 2.9)	(2a) 6.30/−23.06	(2b) 4.26/−23.06
2 (pH 7)	(2a) 5.64/−19.50	(2b) 4.49/−19.50
4	(4a) 5.68/−27.74	(4b) 3.60/−27.74
5[a]	(5a) 6.24/−24.92	(5b) 4.23/−24.92
6[a]	(6a) 6.14/−24.60	(6b) 4.18/−24.60
8	(8a) 3.85/−35.26	(8b) 2.91/−35.26
	(8c) 3.53/−32.55	(8d) 2.79/−32.55
10	(10a) 6.59/−28.96	(10b) −[b]
11[c]	(11a) 6.04/−21.89	(11b) 4.35/−21.89

[a] Assignments for **5** and **6** are ambiguous. [b] Too broad to observe. [c] Phosphate adduct $[(\eta^6\text{-bip})Ru(en)(PO_4)]^-$.

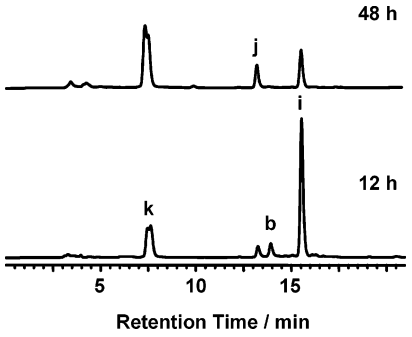


Figure 4. HPLC chromatograms recorded 12 and 48 h after the start of the reaction of complex **1** (20 μM) with a 250-fold molar excess of GSH in phosphate buffer (pH 7) containing 22 mM NaCl at 310 K. Peak assignments: (b) aqua adduct $[(\eta^6\text{-bip})Ru(en)(H_2O)]^{2+}$ (**2**); (i) thiolato adduct **4**; (j) sulfenato adduct **8**; (k) GSH/GSSG (overlapped).

for NMR study. In the 2D $[^1H,^1H]$ COSY (Figure 5) and NOESY (Figure S5) NMR spectra, three sets of proton resonances are observed, assignable to the glutathione, biphenyl, and en groups. The chemical shifts for the former two groups are listed in Tables 2 and S1, respectively. It can be seen that ruthenium coordination induces large changes in the chemical shifts of the Cys β-CH_2, α-CH, and α-NH protons of the thiolate ligand. The signals of two CH_2 protons separate into two quartets (δ 2.96, 2.81), and the resonances of α-CH and α-NH protons are shifted to high field by 0.16 and 0.14 ppm, respectively,

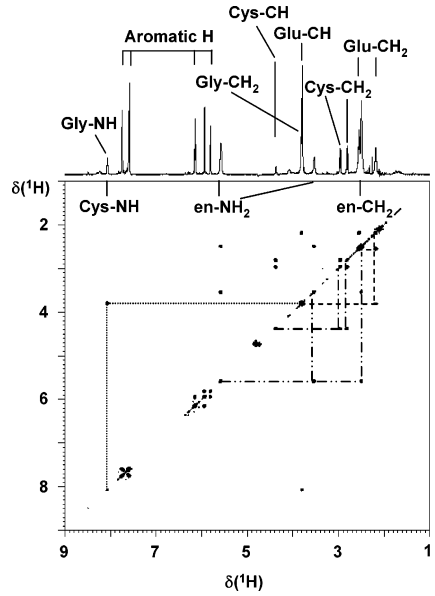


Figure 5. 1D ^1H and 2D $[^1H,^1H]$ COSY NMR spectra for HPLC fraction (i) (see Figure 4) from the reaction of **1** with GSH (5:25 mM) in phosphate buffer (pH 7) containing 22 mM NaCl at 310 K for 24 h.

compared with those of the free peptide. Strong NOEs signals are observed (Figure 6), corresponding to the interaction of the β-CH_2 protons with the protons of the coordinated phenyl ring (A).

HPLC and MS studies (Figure S6) showed that the thiolato adduct **4** is not stable in air at pH 7, but is converted to the sulfenato complex **8**, instead of into the diruthenium thiolato complex $[((\eta^6\text{-bip})Ru)_2(GS)_3]^{2-}$ (**7**) as at pH 3. We observed this instability first over a period of 3 d while NMR spectra of an HPLC-isolated sample of **4** (pH 7) were being recorded; during that time 52%[15] of **4** transformed into **8** (Figure S6). The IR spectrum of this sample showed a band at 1018 cm^{-1} assignable to S═O stretching.

A comparison was also made between the HPLC profiles for reactions carried out under O_2 and under argon (Figure 7). For reactions under O_2, the sulfenato product **8** was dominant, and

(15) Calculated on the basis of HPLC peak areas for adducts **4** and **8**, calibrated by ICP-OES (Table 1).

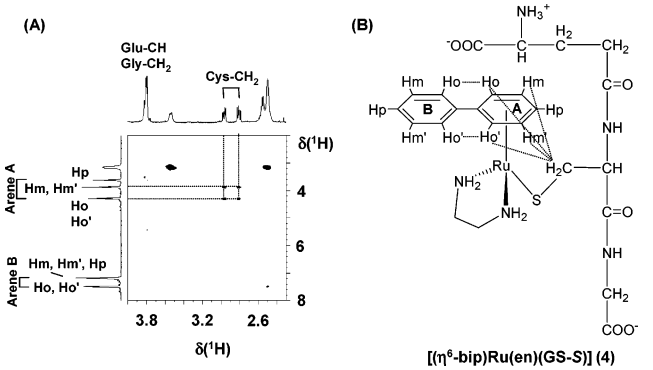


Figure 6. (A) NOEs between the biphenyl and Cys β-CH_2 protons in complex **4**, indicating their proximity (<ca. 5 Å apart). (B) Structure for **4** with NOEs indicated by dotted lines. For NOEs between protons of the two phenyl rings, see Figure S5.

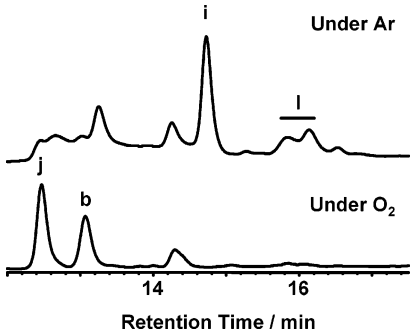


Figure 7. HPLC chromatograms for reactions of complex **1** (20 μM) with a 250-fold molar excess of GSH in phosphate buffer (10 mM, pH 7) containing 22 mM NaCl under O_2 (bottom) or Ar (top) at 310 K for 48 h. Peak assignments: (b) [(η^6-bip)Ru(en)(H_2O)]$^{2+}$ (**2**); (i) thiolato adduct **4**; (j) sulfenato adduct **8**; (l) dinuclear adduct **9**. The formation of the sulfenato adduct **8** is largely suppressed under Ar. For MS data on adduct **9**, see Figure S7 in Supporting Information.

little of **4** remained after 48 h reaction; however, under Ar the thiolato adduct **4** was dominant, and little of **8** was formed. For the reaction under Ar, two new HPLC peaks with retention times of 16.60 and 17.04 min were observed, labeled l in Figure S7. The mass spectrum of fraction l (Figure S7), separated from a mixture of 1 mM **1** and 10 mM GSH in pH 7 buffer containing 22 mM NaCl at 310 K which had reacted for 48 h under Ar, suggests that it may contain a novel diruthenium thiolato/sulfinato adduct (**9**; see Figure S7).

Competitive Reactions of GSH and cGMP with Complex 1. Next we investigated the competitive binding of cGMP and GSH to complex **1** under physiologically relevant conditions. Incubation of 20 μM **1** with 250 mol equiv of GSH and 25 mol equiv of cGMP in phosphate buffer (pH 7) containing 22 mM NaCl at 310 K for 30 h gave rise to both GSH- and cGMP-containing products (Figure 8). All initial solutions at neutral pH were purged with N_2 before and after mixing to minimize their O_2 content. HPLC fractions i and j (Figure 8) contained the thiolato adduct **4** and sulfenato adduct **8**, respectively. The mass spectrum of fraction m (Figure S8) contained a singly charged ion peak at *m*/*z* 774.2 and a doubly charged ion peak at *m*/*z* 659.9, assignable to the TFA ion-paired adduct of the cGMP complex [(η^6-bip)Ru(en)(cGMP)][TFA] (calcd *m*/*z* 774.1 for {[(η^6-bip)Ru(en)(cGMP)][TFA] + H}$^+$) and the dimer {[(η^6-bip)Ru(en)(cGMP)]$_2$}$^{2+}$ (calcd *m*/*z* 660.1), respectively, confirming the formation of the cGMP adduct [(η^6-bip)Ru(en)-

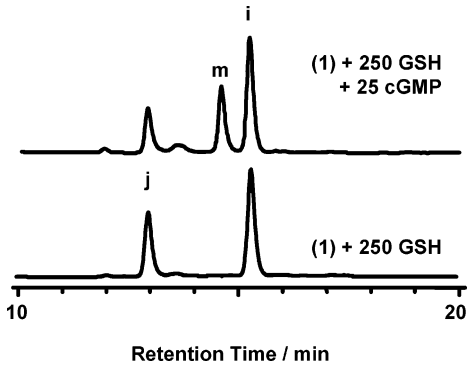


Figure 8. HPLC chromatograms for reactions of **1** (20 μM) with 250 mol equiv GSH (bottom), and with 250 mol equiv GSH and 25 mol equiv cGMP (top) in phosphate buffer (pH 7) containing 22 mM NaCl at 310 K for 30 h. Peak assignments: (i) thiolato adduct **4**; (j) sulfenato adduct **8**; (m) cGMP adduct **10**. The nucleotide adduct is still formed even in the presence of a 10-fold excess of GSH.

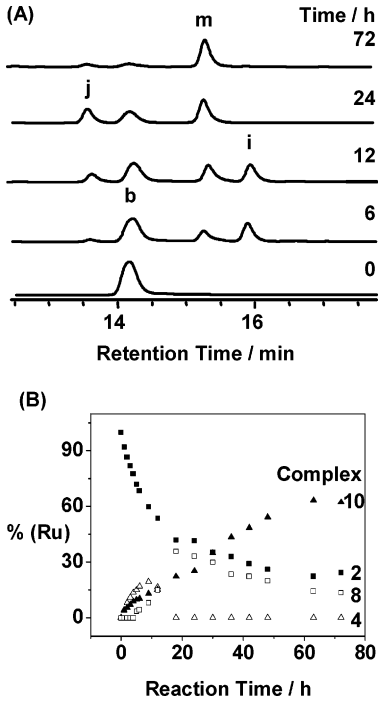


Figure 9. (A) HPLC time-course for the competitive reaction of **1** (20 μM) with 250 mol equiv of GSH and 25 mol equiv of cGMP in phosphate buffer (pH 7) containing 22 mM NaCl at 310 K. Peak assignments: (b) [(η^6-bip)Ru(en)(H_2O)]$^{2+}$ (**2**); (i) thiolato adduct [(η^6-bip)Ru(en)(GS)] (**4**); (j) sulfenato adduct [(η^6-bip)Ru(en)(GS(O))] **8**; (m) [(η^6-bip)Ru(en)-(cGMP)]$^+$ (**10**). (B) Variation of the relative Ru concentrations of species detected during the reaction in (A) with time. The HPLC areas were calibrated by ICP-OES; the relative extinction coefficients for complexes **2**, **4**, **8**, and **10** are listed in Table 1. The thiolato adduct appears to be converted to the sulfenato adduct after 6 h that, in turn, appears to be converted to the cGMP adduct after 24 h. Some formation of **10** by direct reaction of cGMP with **2** may also occur.

(cGMP)]$^+$ (**10**). After 30 h of reaction, the ratios of **4**:**8**:**10** were 36:30:26%. The presence of N7-bound cGMP in **10** was confirmed by the ^{1}H NMR spectrum of a 24-h reaction mixture of 1 mM **1** with 5 mM GSH and 1 mM cGMP, which showed a peak at δ 8.28 assignable to H8 of bound cGMP in **10**, shifted downfield by 0.31 ppm relative to free cGMP.[4b]

A similar reaction mixture was prepared and sampled in air at various time intervals over 3 d for HPLC assays (Figure 9). In this case, conversion of the thiolato adduct **4** to the sulfenato complex **8** began after only 6 h, and by 24 h all the thiolato

adduct had disappeared. After 72 h, the major product (ca. 62% of the total Ru) was the cGMP adduct **10** (Figure 9).

Next we added cGMP (25 mol equiv) to a solution of **1** (20 μM) which had been allowed to react with 250 mol equiv GSH for 6 h under the same conditions as above (phosphate buffer, pH 7, 22 mM NaCl, 310 K). After 24 h, the cGMP product **10** accounted for 12% of the Ru present, the thiolato adduct **4**, 49%, and sulfenato adduct **8**, 34% (Figure S9). Addition of only 5 mol equiv of cGMP to a similar solution of 20 μM **1**, which had reacted with 250 mol equiv of GSH for 6 h, still gave rise to a detectable amount (5%) of the cGMP product **10** after 24 h (Figure S9).

The competitive reaction of GSH and cGMP at millimolar concentrations (**1**:GSH:cGMP 1:5:1 mM) in phosphate buffer (pH 7) containing 22 mM NaCl was also followed by 2D [^{1}H,^{15}N] HSQC NMR at 310 K using ^{15}N-labeled **1** (^{15}N-**1**). The spectra are shown in Figure S10 and the ^{1}H and ^{15}N NMR chemical shifts are listed in Table 3. During the early stages (<1 h), a pair of new cross-peaks (4a, 4b) was observed for the ^{15}N-en ligand in thiolato adduct **4**, and after 2 h, cross-peak 10a appeared, assignable to the cGMP adduct [(η^6-bip)Ru(^{15}N-en)(cGMP)]$^+$ (**10**). This increased in intensity until 72 h. After 12 h, two pairs of cross-peaks (8a/b and 8c/d) were detectable and can be assigned to the sulfenato complex [(η^6-bip)Ru(^{15}N-en)(GS(O))] (**8**) in accordance with the HPLC results (Figure 9). Cross-peaks 4a/b and 8a/b/c/d slowly decreased in intensity after ca. 50 h. It is notable that a pair of cross-peaks (11a/b) assignable to the phosphate adduct [(η^6-bip)Ru(^{15}N-en)-(PO_4)]$^-$ (**11**)[4a,b] is observed for the equilibrium solution of complex **1** in 50 mM phosphate buffer (pH 7). After ca. 36 h, cross-peak 12a corresponding to the release of ^{15}N-en from glutathione-coordinated Ru complexes such as **9** appeared.

Discussion

Reaction of Complex 1 with GSH in Unbuffered Aqueous Solution. Treatment of 0.3 mM complex **1** with 10 mol equiv excess of GSH in unbuffered aqueous solution gave rise to two products (peaks d and e in Figure 1) after just ca. 10 min, and then a third (peak f, Figure 1) after 30 min. The mass spectra showed that all three adducts are monoglutathione complexes (Figure S1), but a fragment ion peak at *m*/*z* 561.9 (calcd *m*/*z* 562.1 for {(η^6-bip)Ru(GS)}$^+$) corresponding to the releasing of en was observed only for fraction f. This suggests that the Ru−N bonds in this complex are weakened as might be the case for an S-bound thiolato complex [(η^6-bip)Ru(en)(GS-*S*)] (**4**) (for NMR identification vide infra). The trans-labilizing effect of sulfur is well-known in Pt^{II} chemistry.[16] The subsequent displacement of the labilized en by glutathione leads to the formation of the diruthenium thiolato adduct [((η^6-bip)Ru)$_2$-$(GS)_3$]$^{2-}$ (**7**) as the major final product (peak g in Figure 1).

1D and 2D [^{1}H,^{1}H] NMR spectra acquired from HPLC fraction g (Figures 1−3 and S2) showed only one set of proton resonances assignable to the two biphenyl and three glutathione ligands and no peaks for en protons. This indicates that the two biphenyl and three glutathione ligands in this adduct are each magnetically equivalent, and that adduct **7** [((η^6-bip)Ru)$_2$-$(GS)_3$]$^{2-}$ is formed via the substitution of en in [(η^6-bip)Ru-(en)(GS-*S*)] (**4**) by two glutathione ligands. Compared to those of glutathione itself, the cysteinyl β-CH_2 and α-CH proton resonances of coordinated glutathione are greatly shifted to high field (0.42−0.58 ppm), as expected for an S-bound glutathione ligand. Therefore, this diruthenium species is assigned as the triply S-bridged thiolato complex [((η^6-bip)Ru)$_2$(GS-μ-*S*)$_3$]$^{2-}$ (**7**). The observed NOEs between the protons of the coordinated phenyl ring (A) and cysteinyl β-CH_2 and α-CH protons of coordinated glutathione provide evidence for the proximity of these protons (Figure 3).

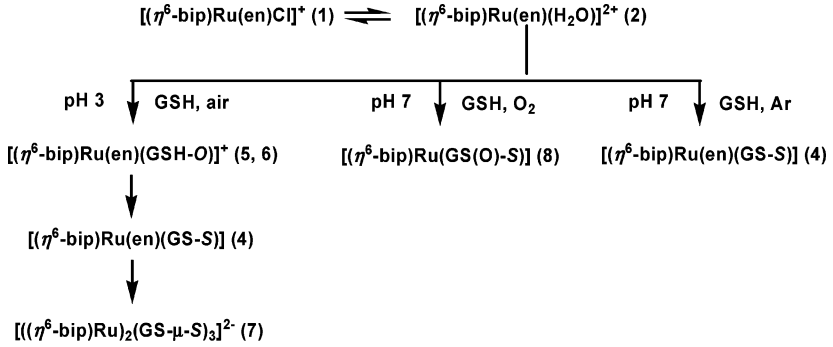
At low pH (ca. 3.0), the amino group of GSH is protonated and unlikely to coordinate to Ru. Therefore, fractions d and e are more likely to correspond to Glu or Gly carboxylate-bound species [((η^6-bip)Ru)(en)(Glu-Cys-Gly-*O*)]$^+$ (**5**) and [((η^6-bip)-Ru)(en)(*O*-Glu-Cys-Gly)]$^+$ (**6**). These two intermediates were not stable long enough in aqueous solution to allow sufficient NMR data to be recorded to provide an unambiguous identification of the binding sites. However, the 2D HSQC NMR spectra for the reaction of 2 mM ^{15}N-**1** with 10 mM GSH in unbuffered aqueous solution over a period of ca. 10 h showed two pairs of cross-peaks corresponding to the formation of the intermediates **5** and **6** (Figure S3), and their ^{15}N-en ^{1}H/^{15}N chemical shifts (Table 3; δ 5a 6.24/−24.9, 5b 4.23/−24.9; 6a 6.14/−24.6, 6b 4.18/−24.6) are in a similar range to those for ^{15}N-en groups in carboxylate-bound adducts of cystine [(η^6-bip)Ru(en)(Cys_2H_2-*O*)]$^{2+}$ (δ 6.07/−24.7, 4.12/−24.7), cysteine [(η^6-bip)Ru(en)(L-CysH-*O*)]$^+$ (δ 6.08/−24.1, 4.08/−24.1)[8] and acetate (δ 6.06/−25.6, 4.13/−25.6).[17]

Chloro Ru^{II} arene complexes such as complex **1** undergo rapid hydrolysis with half-lives ranging from 5 to 10 min.[18] In aqueous solution, in the presence of nucleophiles such as acetate, the aqua ligand is readily displaced.[17,18] Hence, the carboxylate-bound intermediates [(η^6-bip)Ru(en)(Glu-Cys-Gly-*O*)]$^+$ (**5**) and [(η^6-bip)Ru(en)(*O*-Glu-Cys-Gly)]$^+$ (**6**) are kinetically favored products from reaction of **1** with 10 mol equiv of GSH and are formed via displacement of the aqua ligand in [(η^6-bip)Ru(en)-(H_2O)]$^{2+}$ (**2**) (Scheme 1). However, sulfur-coordination of thiolate to {(η^6-bip)Ru(en)}$^{2+}$ appeared to be thermodynamically favored, and the thiolato adduct [(η^6-bip)Ru(en)(GS-*S*)] (**4**) became the dominant product after 6 h. Subsequently, the en ligand of **4** was readily released assisted by en protonation, by the trans-labilizing effect of S-bound glutathione and binding of two other S-bound glutathione ligands, giving the triply S-bridged diruthenium thiolato complex [((η^6-bip)Ru)$_2$(GS-μ-*S*)$_3$]$^{2-}$ (**7**). This reaction pathway is similar to that we observed previously for reaction of L-cysteine with **1**.[8]

Reaction of Complex 1 with GSH under Physiologically Relevant Conditions. To gain insight into the possible role of GSH in the biological activity of Ru^{II} arene anticancer complexes such as **1**, reactions were studied under conditions similar to those which might be present in cells: micromolar Ru concentrations, in pH 7 buffer containing 22 mM NaCl (the cytoplasmic concentration of chloride)[19] and at 310 K. When the O_2 content of the solutions was minimized by purging with N_2 before and after mixing of the reactants, the thiolato adduct [(η^6-bip)Ru(en)(GS-*S*)] (**4**) was dominant for 12 h in reactions of 20 μM **1** with a 250-fold molar excess of GSH (5 mM).

(16) Rau, T.; Alsfasser, R.; Zahl, A.; van Eldik, R. *Inorg. Chem.* **1998**, *37*, 4223−4230.
(17) Wang, F. Y.; Bella, J.; Parkinson, J. A.; Sadler, P. J. *J. Biol. Inorg. Chem.* **2005**, *10*, 147−155.
(18) Wang, F. Y.; Chen, H. M.; Parsons, S.; Oswald, L. D. H.; Davidson, J. E.; Sadler, P. J. *Chem.—Eur. J.* **2003**, *9*, 5810−5820.
(19) Jennerwein, M.; Andrews, P. A. *Drug Metab. Dispos.* **1995**, *23*, 178−184.

Scheme 1. Pathways for Reactions of Complex **1** with GSH at pH **3** and pH **7**



[a] Minor multinuclear products (HPLC fractions h, Figure 1, and l, Figure 7) are omitted.

The thiolato adduct **4** was characterized by 1D ^{1}H and 2D [^{1}H,^{1}H] NMR spectroscopy. The β-CH$_2$ protons appear as two separated quartets, and the α-CH and α-NH proton resonances shift to high field by 0.16 and 0.14 ppm, respectively (Figures 5, 6), relative to the free peptide. A fragment ion MS peak corresponding to release of the en ligand from this complex is observed (Figures S1, S4), suggesting that S-bound glutathione can labilize the en ligand in **4**. Strong NOEs (Figure 6) corresponding to the interaction of the β-CH$_2$ protons with the protons of the coordinated phenyl ring (A) provide further evidence for S-bound glutathione coordination in **4**.

In reactions at pH 7, the thiolato complex **4** was still the main product after 48 h (Figure 4), and there was no loss of the chelated en ligand, nor formation of the diruthenium complex [((η^6-bip)Ru)$_2$(GS-μ-*S*)$_3$]$^{2-}$ (**7**) as occurred in unbuffered aqueous solution. However, unexpectedly after ca. 6 h the reaction gave rise to the sulfenato complex [(η^6-bip)Ru(en)(GS(O))] (**8**), the amount of which increased to 11% of the total Ru after 12 h and 19% after 48 h.

Further studies showed that under strictly anaerobic conditions, the thiolato complex **4** was stable and the dominant final product, and no sulfenato adduct **8** was detectable even after 48 h (Figure 7). In contrast, under an O_2 atmosphere, the sulfenato complex **8** was the dominant final product, and no thiolato complex **4** was detectable after 48 h (Scheme 1). This strongly suggests that the sulfenate arises from oxidation of the thiolato complex and that the oxygen atom in the sulfenate originates from O_2. Sulfenic acids are generally too reactive to isolate,[20] but a few examples are known in which they are stabilized by binding to transition metal ions such as Co^{III},[21] Ni^{II},[22] Ru$^{II/III}$,[23] Rh^{III},[24] and Ir^{III}.[25] The sulfenato complex **8** obtained here is a new example of the stabilization of a sulfenate by coordination to a transition metal. In principle binding of the sulfenate to Ru^{II} could occur through sulfur or oxygen. In the reported X-ray structures of Ru^{II} and Ru^{III} sulfenate complexes, the sulfenate is S-bonded.[23] A similar situation exists for sulfoxides, for which S-bonding is common, although both modes of binding are known.[26] It seems likely that the preferred mode of binding of glutathione sulfenate in [(η^6-bip)Ru(en)(GS(O))] (**8**) is also through sulfur, as suggested by the SO stretching frequency (vide infra).

We observed a slow conversion of the HPLC-isolated thiolato complex **4** to the sulfenato complex **8** in air (Figure S6), supporting the proposed pathway to **8** by oxygenation of **4**. A characteristic infrared S=O stretching frequency of 1018 cm^{-1} was observed for **8**, a frequency similar to those reported for the S-bonded sulfenates in [IrCl$_2$(CO)(PPh$_3$)(S(O)Me)] (1013 cm^{-1})[25] and [CpW(CO)$_3$(CH$_2$S(O)Me)] (1017 cm^{-1})[27] and not nearly so low as the 855 cm^{-1} found for the *O*-sulfinates (ν_{as}(SOIr)) in complex Ir(*O*-O$_2$S-*p*-tolyl)(CO)(PPh$_3$)$_2$,[25] evidence for S coordination in the sulfenate **8**.

Surprisingly, the reaction of 20 μM **1** with 250 mol equiv of GSH at pH 7, under Ar, also gave rise to an apparent dinuclear complex (**9**). MS data (Figure S7) suggest that **9** contains both glutathione (GS^{2-}) and glutathione sulfinate (GSO$_2^{2-}$) as ligands, but further work will be needed to confirm this as well as the oxidation state of Ru. This adduct increased in proportion for reactions of 1 mM **1** with 10 mM GSH under the same conditions (Figure S7), suggesting that the thiolato ligand in complex **4** [(η^6-bip)Ru(en)(GS-*S*)] is oxidized to the sulfinate, with water supplying the oxygen atoms. This would imply that lower oxidation states of Ru (e.g., Ru$^{I/0}$) may be involved. It is notable that Kuhlman and Rauchfuss have observed formation of the mixed oxidation state (Ru^{I}/Ru^{II}) tetranuclear cluster [(*p*-cymene)Ru$_4$S$_3$]$^{2+}$ from reaction of [(*p*-cymene)RuCl$_2$)$_2$] with (Me$_3$Si)$_2$S.[28] Interestingly the non-heme iron enzyme nitrile hydratase contains both cysteine-sulfinic (Cys-SO$_2$H) and cysteine-sulfenic acid (Cys-SOH) residues, formed by posttranslational modification.[29] The sulfenate residue (at Cys114) appears to be essential for enzymatic activity, and oxidation to

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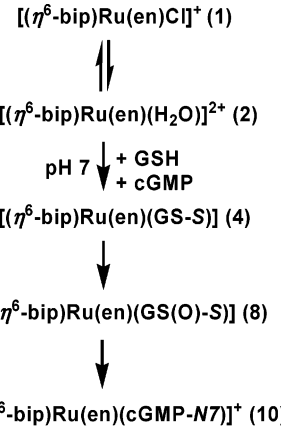
Scheme 2. Pathway for Reaction of Complex **1** with cGMP and GSH at pH **7**[a]



[a] The scheme is not intended to imply that all of **10** is formed via **8**.

a sulfinate (the inserted oxygen being supplied by water) inactivates the enzyme.

Competitive Reaction of GSH and cGMP with Complex 1. In this work, cGMP was chosen as a model nucleotide for investigating competitive reactions with glutathione and complex **1** because it is a diester as are the nucleotides in DNA and RNA. Our previous work showed that en Ru^{II} arene complexes exhibit a high selectivity for N7 of guanine in reactions with DNA[3b,4c] and show little binding to the phosphate group of diesters.[4] The en NH_2 protons in $[(\eta^6\text{-arene})Ru(en)Cl]^+$ can form a strong H-bond with the exocyclic C6 carbonyl oxygen of G but are repulsive toward exocyclic amino groups of nucleobases, and this appears to contribute to the high base-selectivity. Arene ligands which contain an extended π-electron system, as in the biphenyl group of **1**, can provide additional stabilization for the interaction between the Ru^{II} arene complexes and nucleosides or nucleotides by hydrophobic $\pi-\pi$ stacking with the purine ring of G.[4a,b]

Treatment of 20 μM **1** with 250 mol equiv of GSH and 25 mol equiv cGMP[30] under hypoxic and physiologically relevant conditions (pH 7, 22 mM NaCl, 310 K) gave rise to both glutathione adducts (thiolato complex **4** and sulfenato complex **8**) and cGMP adduct $[(\eta^6\text{-bip})Ru(en)(cGMP\text{-}N7)]^+$ (**10**) (Figure 8, Scheme 2). Our preliminary experiments[31] on the reaction of **1** with the 14-mer oligo d(TATGTACCATGATA) show that DNA adducts still form even in the presence of a 25-fold molar excess of GSH. 2D [^{1}H,^{15}N] HSQC NMR spectra recorded over a period of 72 h at higher concentrations, showed cross-peaks corresponding to the formation of these three adducts (Figure S10). No peaks with ^{15}N-en chemical shifts similar to those of *O*-bound adducts **5** and **6** (Figure S3) were observed, again suggesting that **8** contains an S-bound sulfenate.

In the presence of air, the thiolato adduct **4** was oxygenated by dioxygen to the sulfenato complex **8**. The coordinated sulfenato ligand appeared to be readily displaced by cGMP giving the cGMP adduct **10** $[(\eta^6\text{-bip})Ru(en)(cGMP\text{-}N7)]^+$ as the dominant product of the reaction (Figure 9, Scheme 2). This implies that the sulfenato ligand is readily substituted by cGMP, but the thiolato ligand is not. Cisplatin and related Pt^{II} anticancer complexes can reduce disulfide bonds,[32] but thiolates bound to Pt^{II} are not readily displaced by N7-donor ligands, guanine or adenine.[7e,33] However, S-donor thioether ligands such as L-methionine[7b,c] and S-methylated glutathione (GSMe)[7d,34] bound to Pt^{II} are readily substituted by N7-donor nucleosides or nucleotides and oligonucleotides. On the other hand, GSH facilitates the binding of $[Ru^{III}Cl(NH_3)_5]^{2+}$ to DNA through reduction to $[Ru^{II}(H_2O)(NH_3)_5]^{2+}$. However, at [GSH]/[Ru] > 1, GSH inhibits the binding of $[Ru^{III}Cl(NH_3)_5]^{2+}$ to DNA by forming $[Ru^{III}(GS)(NH_3)_5]^{2+}$, and a high [GSH]/[Ru] even eliminates G N7 coordination.[1a] Therefore, the observed oxidation of coordinated glutathione to the sulfenate appears to provide a facile route for displacement of S-bound glutathione by G N7, similar to the methylation of thiol to thioether. A Ru−S(sulfenate) bond would be expected to be weaker than a Ru−S(thiolate) bond. As an example, the Co−S bond length increases from 2.226 Å in the thiolato complex $[(en)_2Co(SCH_2CH_2NH_2)]^{2+}$ to 2.253 Å in the respective sulfenato complex.[21c]

Conclusions

Cells contain high (millimolar) concentrations of the tripeptide glutathione which has several binding sites for metal ions: the amino and carboxylate groups at the Glu terminus, carboxylate at the Gly terminus, and thiolate sulfur of the central Cys residue. Binding to the deprotonated amide nitrogen of Cys is possible when a N/S chelate ring can be formed, as we have observed for platinum amine complexes.[32a] For heavier, "soft" transition metal ions, thiolate sulfur is a particularly strong site, and for Pt^{II}, such bonds are essentially formed irreversibly. Elevation of GSH levels therefore provides cells with detoxification and resistance mechanisms. The aim of this work was therefore to investigate reactions of the organometallic Ru^{II} arene anticancer complex $[(\eta^6\text{-bip})Ru(en)Cl]^+$ with GSH and, in particular, whether the presence of a large molar excess of GSH (as would be the case in cells) could prevent binding to the nucleobase guanine, the predominant binding site for $\{(\eta^6\text{-bip})Ru(en)\}^{2+}$ on DNA.[3b,4c] Our strategy involved separation of the products by HPLC and identification by ESI-MS, and after preparative HPLC, characterization by NMR spectroscopy. ^{15}N-labeling of the chelated ethylenediamine ligand and use of 2D [^{1}H,^{15}N] HSQC NMR spectroscopy was helpful for elucidation of some of the complicated reaction pathways.

First we studied reactions at 310 K (body temperature) in unbuffered aqueous solution since buffers themselves usually contain potential ligands. Hydrolysis of **1** was followed by initial binding to the carboxylate sites and then to the thiolate sulfur

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(30) Concentration chosen on the basis that 10^6 cells contain ca. 10 μg RNA and 5 μg DNA, a guanine content of 0.01 μmol, which is ca. 25× typical intracellular Ru (380 pmol Ru/10^6 cells, for dose of **1** = 25 μM: Wang, F. Y.; Zeitlin, B.; Habtemarian, A.; Eades, L.; Aird, R.; Jodrell, D. I.; Sadler, P. J. unpublished material).

(31) **1**: oligo: GSH = 1:2:50, 48 h, 310 K, 4 mM NaCl, 10 mM phosphate buffer pH 7. Previously we showed that this oligo is ruthenated at the G sites even in the presence of 8 mol equiv of L-histidine (ref 17).

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(Scheme 1), but at this acidic pH (3) displacement of en by further S-bound GSH ligands was favorable, and, after 24 h, the major product was the dinuclear S-bridged complex $[((\eta^6\text{-bip})\text{Ru})_2(\text{GS-}\mu\text{-}S)_3]^{2-}$ (**7**).

Subsequent reactions were studied at pH 7 using phosphate buffer in the presence of a typical cytoplasmic concentration of chloride (22 mM) and at Ru concentrations relevant to cytotoxicity (micromolar). Here, in N_2-purged solutions, the S-bound adduct $[(\eta^6\text{-bip})\text{Ru(en)(GS-}S)]$ (**4**) was the major product, and there was little tendency for loss of the en ligand. However, unexpectedly, complex **4** was very sensitive to the presence of air and underwent oxidation to the sulfenato complex $[(\eta^6\text{-bip})\text{Ru(en)(GS(O)-}S)]$ (**8**).

Under these physiologically relevant conditions, competitive reaction of complex **1** with 250 mol equiv GSH and 25 mol equiv cGMP gave rise to the cGMP adduct $[(\eta^6\text{-bip})\text{Ru(en)-(cGMP-}N7)]^+$ (**10**) as the major product, accounting for ca. 62% of total Ru after 72 h. This suggests that oxidation of coordinated glutathione in the thiolato complex **4** to the sulfenate in **8** provides a facile route for displacement of S-bound glutathione by G N7, a route for RNA and DNA ruthenation even in the presence of a large excess of GSH.

When reactions were carried out in an atmosphere of O_2, the sulfenato complex **8** was the main product, suggesting that the oxygen atom in the sulfenate ligand arises from O_2. Intriguingly, reactions carried out under strictly anaerobic conditions under Ar gave rise not only to the expected thiolato adduct **4** but also to an apparent dinuclear complex containing more highly oxidized GSH (sulfinate, GSO_2^{2-}). This suggests that lower Ru oxidation states may be involved under these conditions and that water can also act as oxygen-atom donor. However, these reactions require further investigation before such pathways can be confidently assigned.

This work has revealed unusual redox reactions of cysteinyl adducts of a monofunctional ethylenediamine Ru^{II} arene complex with glutathione. These could play a significant role in the biological activity of ruthenium arene anticancer complexes. Oxygenation of GSH may itself have biological consequences. It remains to be seen if ruthenium arene complexes can induce oxygenation of cysteine residues in proteins. There is much current interest in the role of protein cysteinyl sulfenates in signal transduction, oxygen metabolism, oxidative stress, and in the regulation of transcription,[20a,35] as well as in both cysteinyl sulfenates and sulfinites in the activity of the enzyme nitrile hydratase.[29]

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Supporting Information Available: HPLC, LC−MS, and NMR data (Table S1, Figures S1−S10). This material is available free of charge via the Internet at http://pubs.acs.org.

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