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Ruthenium(III) Polyaminocarboxylate Complexes: Efficient and Effective Nitric Oxide Scavengers

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The preparation of two Ru^{III} polyaminocarboxylate complexes, AMD6245 and AMD6221, and their nitrosyl analogues, AMD6204, AMD6263, and AMD3689, is described. The compounds are characterized by IR, ES-MS, and ^{13}C and ^{15}N NMR spectroscopy where appropriate and cyclic voltammetry. The crystal structures for AMD6245, AMD6263, and AMD3689 are presented. AMD6245 ($C_{10}H_{14}N_2O_9Ru$) crystallized in the $P2_1/c$ space group with $a = 8.4382(2)$ Å, $b = 8.8304(2)$ Å, $c = 17.6321(4)$ Å, $\beta = 99.603°$, $V = 1295.3(2)$ Å^3, and $Z = 4$. AMD6263 ($C_{10}H_{14}N_3O_{10}Ru$) crystallized in the $P2_1/c$ space group with $a = 9.9043(4)$ Å, $b = 13.1144(3)$ Å, $c = 12.0914(4)$ Å, $\beta = 100.191°$, $V = 1545.8(5)$ Å^3, and $Z = 4$. AMD3689 ($C_{14}H_{24.56}N_4O_{13.28}Ru$) crystallized in the $P\bar{1}$ space group with $a = 8.838(2)$ Å, $b = 9.452(3)$ Å, $c = 13.419(4)$ Å, $\alpha = 78.413(6)°$, $\beta = 75.804(6)°$, $\gamma = 73.562(6)°$, $V = 1031.8(5)$ Å^3, and $Z = 2$. The reaction of AMD6245 and AMD6221 with nitric oxide is investigated using EPR spectroscopy and stopped flow kinetics. Upon reaction with NO, a linear, diamagnetic $\{RuNO\}^6$ complex is formed. The substitution reaction of AMD6245 with NO proceeds with a second-order rate constant of 2.24×10^7 M^{-1} s^{-1} at 7.3 °C (pH = 7.4; 50 mM phosphate buffer). The substitution reaction of AMD6221 with NO proceeds with a second-order rate constant of 3×10^5 M^{-1} s^{-1} at 20 °C (pH = 7.4; 50 mM phosphate buffered saline). The NO scavenging ability was assessed using a RAW264 murine macrophage assay by measuring the difference in nitrite produced between untreated control cells and treated cells. At 100 μM AMD6245 has $[NO_2^-] = 12.5$ μM less than the untreated cells and AMD6221 has $[NO_2^-] = 37.6$ μM less than the untreated cells. There is an insignificant difference in the amount of nitrite produced between AMD6263 or AMD3689 treated cells and untreated cells.

Introduction

Nitric oxide (NO) was voted molecule of the year by *Science* in 1992. Subsequently in 1998, Robert Furchgott, Ferid Murad, and Louis Ignarro were awarded the Nobel Prize in physiology or medicine for their contributions to and discoveries of NO acting as a signaling molecule in biological systems. It is not surprising then, that a significant amount of research has been devoted to NO and its role in the physiology and pathophysiology of disease.

NO is produced during the conversion of L-arginine to L-citrulline, a process catalyzed by the enzyme nitric oxide synthase (NOS).[1] There are several isoforms of NOS, and these are divided into the Ca^{2+}-dependent (constitutive NOS (cNOS): nNOS and eNOS) and Ca^{2+}-independent (inducible NOS: iNOS) groups. A dysfunction in NO metabolism has been implicated in many disease states. For instance a decrease in NO production (from cNOS) can lead to severe hypertension, a disease state that is treated by vasodilators (NO donors) such as nitroprusside.[2] Alternatively, an up-regulation of iNOS leading to an overproduction of NO has been shown to play a role in sepsis and inflammatory disorders such as rheumatoid arthritis,[3] inflammatory bowel disease,[4] and asthma.[5] NO has also been implicated to play

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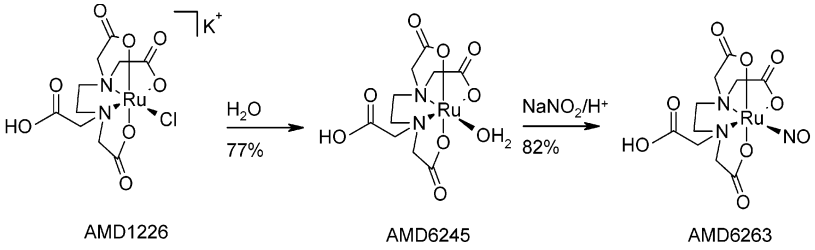
Scheme 1. Preparation of AMD6245 and AMD6263



a role in angiogenesis and tumor progression.[6,7] One approach for the attenuation of NO-mediated diseases is via the inhibition of NOS. In this case, the inhibitor must be selective for iNOS, to prevent any deleterious effects of inhibiting the essential constitutive NOS.

An alternative therapeutic strategy is the use of nitric oxide scavengers. Our program has focused on the use of ruthenium complexes and in particular ruthenium polyaminocarboxylate complexes. An effective NO scavenger must meet certain criteria including (but not limited to) the following: (1) fast NO reaction kinetics; (2) activity and stability in in vitro and in vivo biological systems; (3) low toxicity; (4) rapid clearance from the organism. The formation of nitrosyl complexes[8] is a marked feature of ruthenium chemistry, and formation of the Ru−NO bond stabilizes the ligand trans to the NO molecule. Additionally, the Ru−NO bond itself is extremely stable, persisting through a variety of both redox and substitution reactions. The rich coordination chemistry of ruthenium allows for fine-tuning of the all the necessary properties for an effective NO scavenger listed above through manipulation of the ligand framework.

The advantages to using a NO scavenger over a NOS inhibitor include the nonrequirement of enzyme specificity. In the case of the ruthenium complexes the compartmental localization of the NO scavenger can be controlled with the multidentate ligand framework, adjusting lipophilicity and charge accordingly. The rate of NO scavenging, assuming a second-order process, would also be dependent upon both the concentration of NO and the scavenger. This means that when NO concentrations are elevated, as occurs in a number of disease states, scavenging would be promoted. This kinetic argument does not apply in the case of enzyme inhibitors that are not influenced by NO concentration and therefore inhibit NO synthesis equally in regions with high and low rates of NO synthesis. Here we present the background chemistry of our ruthenium-based NO scavengers, AMD6245 [Ru^{III}(Hedta)OH_2] and AMD6221 [Ru^{III}(H_3dtpa)Cl], underlying the aforementioned criteria.

Synthesis

It has been shown that AMD1226 exists in aqueous solution as AMD6245[9,10] (see Scheme 1); however, this complex has not been isolated as a pure solid in the past. Potassium ion is not a pharmaceutically acceptable ion; therefore, it was necessary to prepare a complex that did not contain this counterion. We decided the best approach would be to remove the necessity of a counterion altogether and prepare the aqua complex. AMD6245 was prepared from AMD1226[11] by heating AMD1226 at reflux in aqueous solution as shown in Scheme 1. The neutral complex AMD6245 precipitates out of the reaction mixture upon cooling and is easily isolated by filtration in >75% yield. A necessary step in this synthesis is the seeding of the mother liquor to induce precipitation. If the seeding step is omitted, the complex stays in solution for several weeks and cannot be isolated. Throughout the synthesis of the complex, ruthenium remains in the +3 oxidation state. AMD6245 was characterized by elemental analysis, electrospray ionization mass spectrometry, and infrared spectroscopy. To confirm that no chloride remained in the complex, the solid was also analyzed for Cl. The most striking feature in the IR spectrum of AMD6245 (compared to AMD1226) is the absence of the Ru−Cl stretch at 299 cm^{-1}. The spectrum also shows strong stretching frequencies assigned as the coordinated carboxylate group (CO_2^- ; 1652 cm^{-1}) and the uncoordinated carboxylic acid (CO_2H; 1741 cm^{-1}); however, these are indistinguishable from the starting material. The electrospray ionization mass spectrum, collected in the negative ion mode has an ion at *m*/*z* 390 assigned as the AMD6245 parent complex with the loss of the water molecule and the loss of one proton [AMD6245 − OH_2 − H]$^-$. The major ions observed upon fragmentation of the *m*/*z* 390 ion are those indicating sequential loss of carboxylate groups (44 mass units) with corresponding ions at *m*/*z* 346 and 302. All ions in the mass spectrum have isotope distribution patterns that agree with the calculated patterns.

AMD6221 was prepared by heating a mixture of K_2-[$RuCl_5(OH_2)$] and H_5dtpa at reflux in 1 mM HCl as shown in Scheme 2. In these slightly acidic conditions AMD6221 exists as a neutral complex enabling facile precipitation from the reaction mixture as a bright yellow solid. This complex has been prepared on up to a 150 g scale without encountering any problems, other than isolation of a higher yield. A

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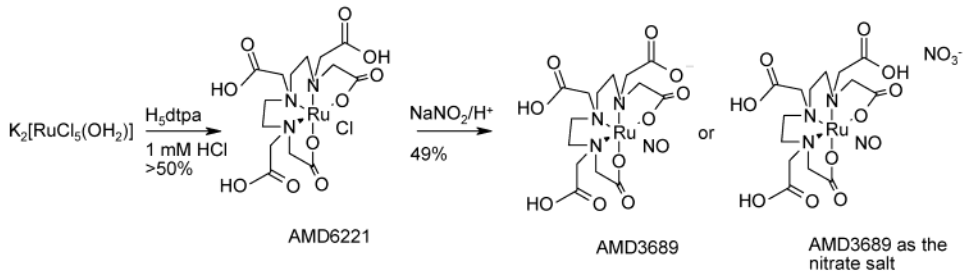
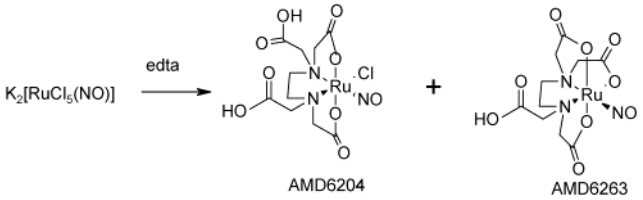
Scheme 2. Preparation of AMD6221 and AMD3689



similar complex, K[Ru(H_2dtpa)Cl], was prepared previously;[12,13] however, as this is the potassium salt it was not suitable for pharmaceutical purposes. AMD6221 was characterized by standard analytical techniques including elemental analysis, IR spectroscopy, and electrospray ionization mass spectrometry. Because the method of preparation is similar to that reported for K[Ru(H_2dtpa)Cl],[12,13] a potassium analysis using inductively coupled plasma mass spectrometry (ICP-MS) was performed. The results of this test confirmed that neither potassium nor any other cation is present and that the formulation of AMD6221 is as suggested. It is possible that the original material obtained with this synthesis was given the incorrect formulation.[12] However, in a more recent report[13] the data available support the formulation of the potassium salt, K[Ru(H_2dtpa)Cl], although Cl and K analyses were not performed. The IR spectrum of AMD6221 has the expected CO_2H stretch at 1710 cm^{-1} and CO_2^- stretch at 1667 cm^{-1} as well as a Ru−Cl stretch at 324 cm^{-1}. The mass spectrum, collected in the negative ion mode, has an ion at *m*/*z* 491 assigned as $[M - Cl - 2H]^-$. The observed isotopic distribution pattern matches that of the calculated pattern. MS/ MS fragmentation of the ion at *m*/*z* 491 shows sequential loss of three carboxylate groups with corresponding ions observed at *m*/*z* 447, 403, and 359.

The nitrosyl analogues of AMD6245 and AMD6221 were prepared to use as negative controls and to develop analytical techniques for the biological assays. The preparations of AMD6263 and AMD3689 are shown in Schemes 1 and 2, respectively. Addition of sodium nitrite to an acidic solution of AMD6245 or AMD6221 resulted in isolation of the nitrosyl complexes AMD6263 and AMD3689 in reasonable yields. Alternate methods previously reported in the literature[11] were originally used in the preparation of the nitrosyl complexes. For instance, the preparation of AMD6204[11] is shown in Scheme 3. In our hands, both AMD6204 and AMD6263 were obtained using this method. In general, the amount of each nitrosyl complex isolated depended on the time of the reaction. A shorter reaction time yielded more of AMD6204, and a longer reaction time produced more of AMD6263. The two compounds could be separated on a Dowex cation (H^+) exchange column where the fractions were monitored using UV−vis spectroscopy. Since there are only subtle differences in the electronic spectrum of the two

Scheme 3. Preparation of AMD6263 and AMD6204



compounds, identification of each compound was difficult. We have found that the best approach to the synthesis of the ruthenium nitrosyl complexes was via the use of sodium nitrite in acidic media. This method works well provided that a pH < 2−3 is maintained during the reaction. Less acidic conditions resulted in the isolation of mixtures containing the product and/or their nitrate salts (see Scheme 2).

The three nitrosyl complexes AMD6204, AMD6263, and AMD3689 were characterized by elemental analysis, IR spectroscopy, electrospray ionization mass spectrometry, and ^{13}C NMR spectroscopy. The most striking feature in the IR spectrum of each of the ruthenium nitrosyl complexes was the presence of a strong stretching frequency centered at approximately 1900 cm^{-1} assigned as the NO stretch shown in Table 5. This is characteristic of a linear $Ru^{II}-{}^+NO$ bond, which agrees well with the crystal structure results for AMD6263 and AMD3689 (vide supra). The ES-MS for AMD3689 detected in the positive ion mode has an ion at *m*/*z* 523 assigned as the $[M + H]^+$ ion. Fragmentation of this ion shows the loss of the coordinated nitrosyl group and then a carboxylate group as shown in Table 1. In the negative ion detection mode an ion at *m*/*z* 521 ($[M - H]^-$) is observed in the ES-MS. Fragmentation of this ion does not show the loss of the coordinated nitrosyl group but only loss of a carboxylate group. The ES-MS for AMD6263 was run in the negative ion detection mode, and an ion at *m*/*z* 420 was observed and assigned as the $[M - H]^-$ ion. Fragmentation of this ion gives ions corresponding to fragments shown in Table 1. An ion corresponding to the loss of the coordinated nitrosyl is observed, as well as ions corresponding to the loss of sequential carboxylate groups. Ions are not observed for the loss of both the coordinated nitrosyl and carboxylate group as was seen with AMD3689. AMD6204 in the positive ion detection mode has an ion at *m*/*z* 480 assigned as the $[M + Na]^+$ ion and also an ion was observed at *m*/*z* 458 which corresponds to the $[M + H]^+$ ion. Both ions have isotope distribution patterns that are in agreement with the calculated patterns. The nitrosyl complexes can also be

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Table 1. Mass Spectral Fragmentation Patterns for Ruthenium Nitrosyl Complexes

AMD3689 (^{15}N)[a]				AMD6263 (^{15}N)[a]	
ion (−ve)	assgnt	ion (+ve)	assgnt	ion (−ve)	assgnt
521 (522)	$[M - H]^-$	523	$[M + H]^+$	420 (421)	$[M - H]^-$
477 (478)	$[M - H - CO_2]^-$	493	$[M + H - NO]^+$	390 (391)	$[M - NO - H]^-$
		449	$[M + H - NO - CO_2]^+$	376 (377)	$[M - H - CO_2]^-$
				332 (333)	$[M - H - 2CO_2]^-$
				288 (289)	$[M - H - 3CO_2]^-$

[a] Data given in parentheses correspond to the analogous ^{15}N nitrosyl complex.

characterized using NMR spectroscopy since they are diamagnetic. The ^{1}H NMR spectra were complex; however, useful structural information can be obtained from the ^{13}C NMR spectra. In the case of AMD3689, there is no symmetry observed in the solution structure (i.e. individual carbon atoms are observed in the ^{13}C NMR spectrum for a total of 14 resonances). Similar results were observed for AMD6263, with a total of 10 carbon atom resonances observed in the ^{13}C NMR spectrum. AMD6204 has a complex ^{13}C NMR spectrum with at least 10 resonances observed in the carbonyl region alone. This suggests that a mixture of structural isomers are obtained during the synthesis of this compound that are not separated on the ion exchange column or that there is significant dissociation of the Cl^- anion at low concentrations, leading to more than one species present in solution, as previously suggested.[11] The difficulties encountered in the isolation of AMD6204 may in fact be explained by this rapid dissociation of Cl^-. The ^{15}NO analogues of AMD6263 and AMD3689 were also prepared as shown in Scheme 1 and Scheme 2 using $Na^{15}NO_2$ as the source of NO. There is a significant difference in the infrared spectrum between the ^{14}N and ^{15}N complexes as expected. The ^{15}NO stretch is shifted by 16−28 cm^{-1} to a lower frequency. The ^{15}N NMR spectra were also recorded for the ^{15}N-enriched nitrosyl complexes of AMD3689 and AMD6263. Both complexes have a single resonance in the ^{15}N NMR in the δ −15 ppm region (referenced to CD_3NO_2), and this along with the ^{13}C NMR data supports the existence of only one structural isomer of both AMD3689 and AMD6263. The ^{15}N chemical shift has also been used as an indicator of the geometry of the coordinated nitrosyl ligand.[14] Typically, bent M−NO bonds are more deshielded than the linear M−NO bonds, and as a result, the bent ^{15}N chemical shifts occur in the range of δ 975−350 and linear ^{15}N chemical shifts occur in the range of δ 0 to −100. Both AMD6263 and AMD3689 have ^{15}N chemical shifts characteristic of linear M−NO bonds.

X-ray Crystallography

A perspective ORTEP drawing of AMD6245 is shown in Figure 1 with an atom-numbering scheme. Selected bond lengths and bond angles are given in Table 2. The structure verifies the pentadentate coordination of the edta ligand, with two nitrogen atoms and three carboxylate groups coordinated to the ruthenium center. A water molecule occupies the sixth coordination site of the ruthenium center. This verifies the

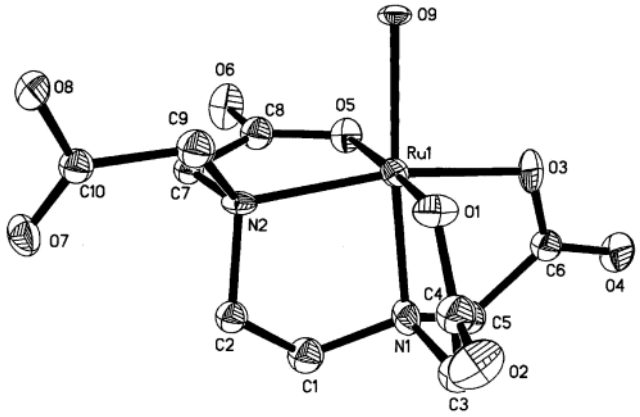

Figure 1. ORTEP drawing of AMD6245. H atoms have been omitted for clarity.

Table 2. Selected Bond Lengths (Å) and Angles (deg) for AMD6245

Ru(1)−O(1)	1.993(7)	O(9)−Ru(1)−N(1)	175.4(4)
Ru(1)−O(3)	2.018(7)	O(3)−Ru(1)−N(2)	162.7(3)
Ru(1)−O(5)	2.062(6)	O(1)−Ru(1)−O(5)	173.6(3)
Ru(1)−O(9)	2.115(12)	O(9)−Ru(1)−O(3)	95.4(4)
Ru(1)−N(1)	2.036(9)	O(9)−Ru(1)−O(1)	91.0(4)
Ru(1)−N(2)	2.124(8)	O(9)−Ru(1)−O(5)	87.3(4)
		O(9)−Ru(1)−N(2)	97.8(4)

structure that is proposed in aqueous solution, which also has a pendant carboxylate group.

The X-ray crystal structures of NH_4[Ru(Hedta)Cl][15] and K[Ru(Hedta)Cl][16] have been reported previously. In both these structures and the structure of AMD6245 the N atom (i.e. N1) trans to the monodentate ligand, in this instance a water molecule and in previous structures a Cl^- ion, has a Ru−N1 bond distance significantly shorter (2.036 Å) than the other Ru−N2 bond length (2.124 Å). This has been attributed to the fact that N1 is involved in three chelate rings to the metal atom drawing it closer to the metal, whereas N2 is only involved in two chelate rings with the metal atom. A slightly distorted octahedral geometry around the Ru center is consistent in this structure and in the similar reported structures. The Ru−O bond lengths vary in the range of 1.993−2.062 Å, which are also in agreement with NH_4[Ru(Hedta)Cl] (2.008−2.072 Å) and K[Ru(Hedta)Cl] (2.007−2.067 Å). In contrast, the longest Ru−O (Ru−O5) distance in the structure of AMD6245 is not the bond trans to the amino group (Ru−O3) as reported for NH_4[Ru(Hedta)Cl].

A perspective ORTEP drawing of AMD6263 is shown in Figure 2. Selected bond lengths and angles are given in Table

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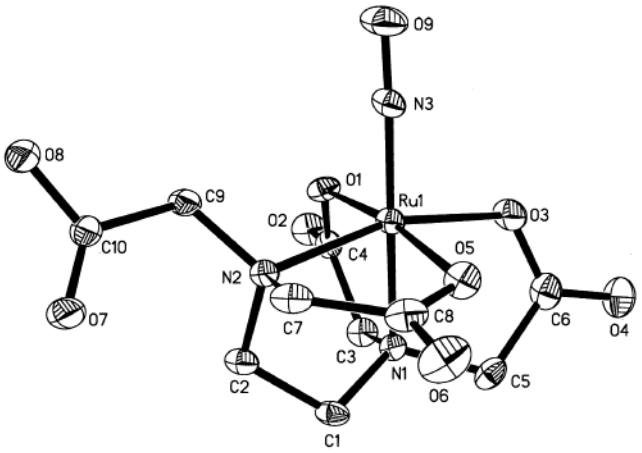

Figure 2. ORTEP drawing of AMD6263. H atoms have been omitted for clarity.

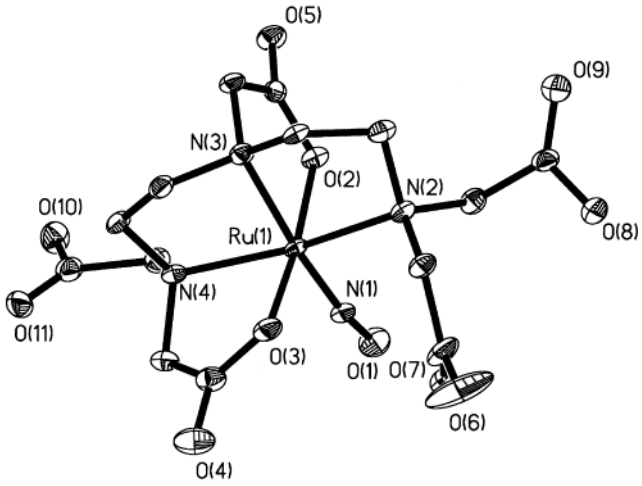

Figure 3. ORTEP drawing of AMD3689. H atoms have been omitted for clarity.

Table 3. Selected Bond Lengths (Å) and Angles (deg) for AMD6263

Ru(1)−O(1)	2.039(4)	N(3)−Ru(1)−N(1)	172.56(19)
Ru(1)−O(3)	2.050(4)	O(3)−Ru(1)−N(2)	162.56(18)
Ru(1)−O(5)	2.004(4)	O(1)−Ru(1)−O(5)	170.22(17)
Ru(1)−N(1)	2.088(5)	N(3)−Ru(1)−O(3)	98.0(2)
Ru(1)−N(2)	2.119(4)	N(3)−Ru(1)−O(1)	91.5(2)
Ru(1)−N(3)	1.749(5)	N(3)−Ru(1)−O(5)	98.2(2)
N(3)−O(9)	1.144(7)	N(3)−Ru(1)−N(2)	99.0(2)
		O(9)−N(3)−Ru(1)	172.5(5)

3. This structure again clearly demonstrates the pentadentate coordination of edta with one pendant carboxylate group. A molecule of NO occupies the sixth coordination site of the ruthenium center. The distinguishing feature of this structure is that the NO is coordinated in an essentially linear fashion with an O−N−Ru bond angle of 172.5° (in agreement with the ^{15}N NMR data). This implies that the complex can be considered in a $Ru^{II}-{}^{+}NO$ formal oxidation state, where the NO transfers an electron to the Ru^{III} center. In this complex, the ruthenium atom is pulled slightly out of the equatorial plane toward the nitrosyl ligand.

A perspective ORTEP drawing of AMD3689 is shown in Figure 3. Selected bond lengths and angles are given in Table 4. The dtpa ligand, like the edta ligand, also coordinates in a pentadentate fashion but with three nitrogen atoms and two

Table 4. Selected Bond Lengths (Å) and Angles (deg) for AMD3689

Ru(1)−O(2)	2.040(3)	N(3)−Ru(1)−N(1)	168.43(16)
Ru(1)−O(3)	2.024(3)	N(4)−Ru(1)−N(2)	163.91(15)
Ru(1)−N(1)	1.764(4)	O(2)−Ru(1)−O(3)	173.61(13)
Ru(1)−N(2)	2.151(4)	N(1)−Ru(1)−O(2)	87.49(15)
Ru(1)−N(3)	2.094(3)	N(1)−Ru(1)−O(3)	98.60(16)
Ru(1)−N(4)	2.129(4)	N(1)−Ru(1)−N(2)	97.57(16)
N(1)−O(1)	1.142(5)	N(1)−Ru(1)−N(4)	97.24(17)
		O(1)−N(1)−Ru(1)	167.0(4)

carboxylate groups occupying five coordination sites of the pseudooctahedral ruthenium center. A NO molecule occupies the sixth coordination site. It is interesting to note that the nitrogen atoms of the dtpa ligand coordinate in a meridonal geometric arrangement around the metal center. The carboxylate group of the central amine and two carboxylate groups from one terminal amine are coordinated to the ruthenium center, leaving two carboxylate groups on the other terminal amine as pendant functional groups. Hence, the dtpa ligand does not coordinate to the ruthenium atom in a symmetrical manner, a structural feature that agrees with the solution NMR structure. The central amine and the coordinated nitrosyl group occupy the axial positions of the pseudooctahedron. The NO also coordinates in a slightly distorted linear fashion; the bond angle of 167.0° is slightly less linear than the coordinated nitrosyl ligand in the corresponding Ru−edta complex (AMD6263).

EPR

The EPR spectra of AMD6221 before and after the addition of NO gas are shown in Figure 4. As demonstrated, on purging of a solution of AMD6221 with NO gas, the paramagnetic Ru^{III} signal disappears as the complex becomes a diamagnetic $\{RuNO\}^6$ complex (Enemark and Feltham classification). This spectrum provides further confirmation, along with the solid-state structure and the ^{15}N NMR data for AMD3689, that AMD6221 reacts with NO to produce a metal nitrosyl complex best described as containing an essentially linear Ru−NO bond. A similar observation in the EPR spectrum was made upon the addition of NO gas to an aqueous solution of AMD1226 (see Supporting Information).

Electrochemistry

The electrochemistry of the Ru complexes and their corresponding nitrosyl analogues was studied under conditions similar to those reported in the literature for H[Ru(edta)(OH_2)].[17] Cyclic voltammograms (Ag/AgCl; Pt wire) were measured for AMD6245, AMD6221, AMD6263, and AMD3689. All solutions were in sodium acetate (0.2 M) buffered to pH 5.5. The results are given in Table 5.

AMD6245 is more stable as the Ru^{III} complex (over the Ru^{II}) compared to AMD6221 with a reduction potential of −200 mV (AMD6245) vs −125 mV for AMD6221. Both AMD6221 and AMD6245 are oxidized at high positive potentials of +860 mV (AMD6221) and +973 mV (AMD6245). This oxidation is electrochemically nonreversible in both compounds, and in the case of AMD6245 this observed oxidation has been attributed to the formation of a

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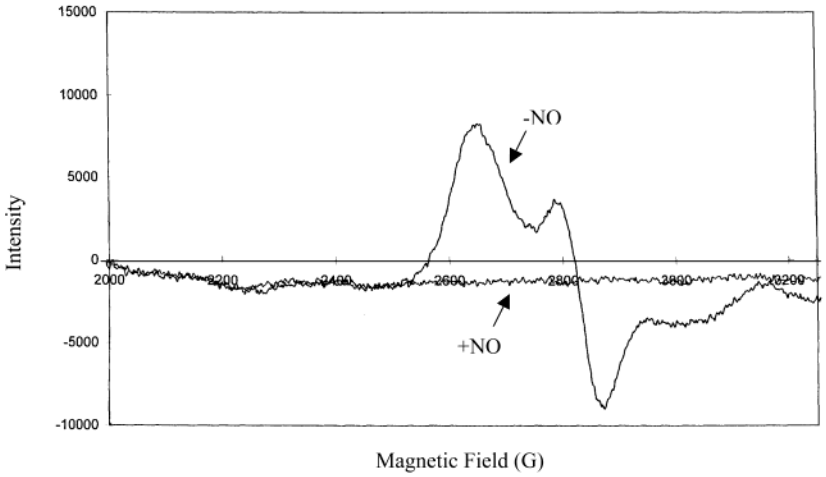

Figure 4. EPR spectrum (10 K) of AMD6221 (100 μM) prepared in sodium phosphate (50 mM) buffered to pH = 7.4 before and after addition of NO gas.

Table 5. Electrochemical Data (mV) and ν(NO) (cm^{-1}) for NO Scavengers

compd	redn[a]	oxidn	ν(NO)
AMD6245	−200 (Ru^{III}/Ru^{II})	+973 (nonreversible)	
AMD6221	−125 (Ru^{III}/Ru^{II})	+860 (nonreversible)	
AMD6263	−300 ($^{+}$NO/NO)		1896; 1880 (^{15}N)
AMD3689	−334 ($^{+}$NO/NO)		1913; 1885 (^{15}N)

[a] The reductions are reversible.

$Ru^{III}-Ru^{IV}$ μ-oxo dimer.[17] Both AMD6263 and AMD3689 have one reduction wave in the −300 mV range, which is due to the reduction of $^{+}$NO to NO as shown in eq 1. There is no oxidation wave observed for these compounds.

$$[Ru^{II}(L)NO]^{3+} + e^{-} \rightleftharpoons [Ru^{II}(L)NO]^{2+} \qquad (1)$$

Kinetics

A saturated nitric oxide aqueous solution was prepared by subjecting a degassed buffer solution to a nitric oxide atmosphere (generated from sulfuric acid and sodium nitrite) and agitating to ensure saturation. The concentration of nitric oxide in the solution was measured by a titration with deoxyferrous myoglobin.[1,18] In general the nitric oxide solutions were on the order of 1.6−2.0 mM.

The kinetics of the reaction between the ruthenium polyaminocarboxylate complexes and NO were investigated using stopped flow kinetic techniques. The reaction of both AMD1226 and AMD6245 with NO has been communicated previously.[19] AMD1226 and AMD6245 exist as the same complex in aqueous solution—a solvent water molecule rendering AMD6245 rapidly substitutes the coordinated Cl^{-} anion of AMD1226.[10] The subsequent reaction with NO (substitution of the coordinated water molecule with NO) is extremely fast with a second-order rate constant of 2.24×10^{7} M^{-1} s^{-1} at 7.3 °C (pH = 7.4; 50 mM phosphate buffer). It has been reported[9,20] that the extreme lability of the coordinated water molecule and fast associative (or I_a) substitution of the coordinated water molecule are direct effects of the pendant carboxylate group. Interaction of the carboxylate group with the coordinated water molecule creates an "open" area for site of attack of the incoming nucleophile.

The addition of an aqueous solution of NO to AMD6221 (pH = 7.4; 50 mM PBS) resulted in an immediate spectral change (Figure 5). An isosbestic point at 278 nm was observed with small increases in absorbance at wavelengths below 270 nm and a small decrease in absorbance at wavelengths above 280 nm. A titration of AMD6221 with NO describes the formation of the corresponding ruthenium nitrosyl complex in a 1:1 stoichiometry. A binding constant, $K_B = 2 \times 10^{5}$ M^{-1}, was determined for the Ru−NO complex. This binding constant is considerably lower than that of AMD6245 (or AMD1226) and NO ($K_B > 10^{8}$ M^{-1}) indicating that the affinity of AMD6221 for NO is lower than that of AMD6245.

The kinetics of the reaction of AMD6221 with NO was followed at two different wavelengths, 260 and 350 nm. The binding of NO by AMD6221 is a second-order reaction with a rate constant of $k = 3 \times 10^{5}$ M^{-1} s^{-1} (pH = 7.4; 50 mM PBS, 20 °C). Taken together with the binding constant, k_{off} (off-rate) for AMD3689 can be calculated as ~1 s^{-1}.

Biological Results

The NO scavenging ability of the ruthenium complexes was evaluated using RAW264 murine macrophage cells. These cells are stimulated to produce NO with the addition of lipopolysaccharide (LPS) and interferon-γ (IFN-γ). The amount of NO produced is estimated after 18 h using the Griess assay,[21] which actually measures the amount of nitrite (an oxidation product of NO in aqueous solution[22,23]). The cells are incubated in the absence (control) or presence of ruthenium complex. The net change in the amount of nitrite accumulated in the media in the treated cells compared to

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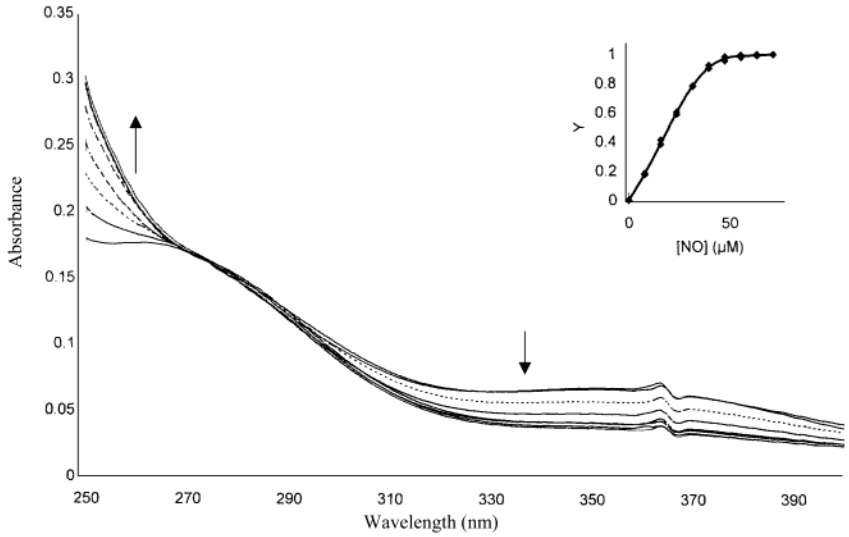

Figure 5. Spectrophotometric change observed during the titration of AMD6221 with NO. The inset is the calculated binding curve of AMD6221 and NO at 350 nm where $Y = (A - A_0)/(A_\infty - A_0)$, the fractional saturation.

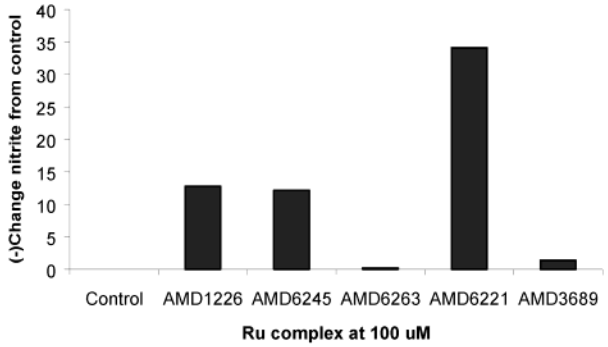

Figure 6. Negative $\Delta[NO_2^-]$ in treated RAW264 cells from the control.

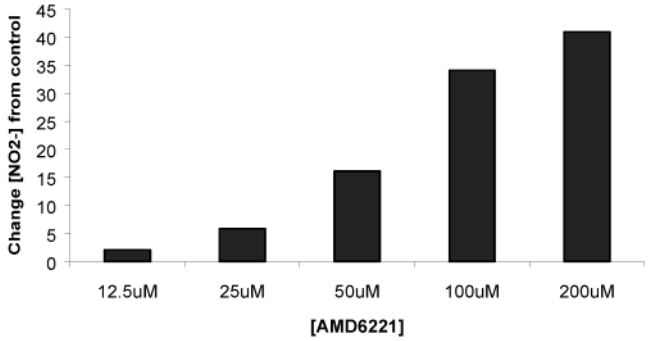

Figure 7. Dose response of AMD6221-treated RAW264 LPS/IFN-γ activated cells.

the untreated cells is an indication of the NO scavenging ability of the compounds being tested. Figure 6 compares the nitrite levels in media from cells treated with various ruthenium complexes at 100 μM depicted as a net negative change in $[NO_2^-]$ from the control. Both AMD1226 and AMD6245 lower the concentration of nitrite in the media by about 12.5 μM compared to the activated cells (control). As we would expect, these two compounds have a similar effect on the nitrite concentration since it has been demonstrated that in aqueous solution these two compounds are identical. AMD6221 has a more significant effect on the level of nitrite accumulation in the cells—a reduction of >30 μM in nitrite from the control is consistently observed. A significant result is that both nitrosyl analogues AMD6263 and AMD3689 do not lower the nitrite level from the control. This supports the theory that the Ru complexes AMD1226, AMD6245, and AMD6221 are acting by scavenging NO and that only one molecule of NO can be scavenged per molecule of ruthenium complex in a biological setting as well as in the chemical setting. Figure 7 demonstrates a dose response observed for the treatment of the activated RAW264 cells with AMD6221. As one would expect, a concentration dependent effect of NO scavenging is observed for AMD6221. There is not a significant difference in the change in nitrite levels between the cells treated with 100 μM and 200 μM AMD6221. This is a direct result of the amount of NO produced by the stimulated cells. Typically, the difference in nitrite levels in media between activated and nonactivated cells is 40−50 μM, so almost the total amount of nitrite produced is being scavenged by 100 μM AMD6221.

Conclusion

We have described the synthesis and characterization of two ruthenium-based NO scavengers, AMD6245 and AMD6221. Both complexes have been completely characterized by standard analytical techniques, including X-ray crystallography for AMD6245. These complexes react rapidly with NO in aqueous solution, to form inert ruthenium nitrosyl complexes, described as $\{RuNO\}^6$ type complexes according to the Enemark and Feltham classification system. All analytical data support the formation of linear Ru−NO complexes. AMD6245 and AMD6221, but *not* their nitrosyl counterparts (AMD6263 and AMD3689), have an impact on the amount of nitrite accumulation in cell media in the RAW264 murine macrophage assay (estimation of the NO scavenging ability). We have previously reported the use of Ru^{III} polyaminocarboxylates in different disease states including sepsis,[21,24] allograft rejection,[25] and cancer.[26] This paper confirms the proposed mechanism of NO scavenging

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by Ru^{III} polyaminocarboxylates in different disease states, through the formation of inactive Ru^{II} nitrosyl ({RuNO}^{6}) complexes.

Experimental Section

Materials. All chemicals were purchased from Aldrich (reagent grade) and used without further purification unless indicated otherwise. K[Ru(Hedta)Cl]·$2H_2O$,[11] $K_2[RuCl_5(NO)]$,[27] and $K_2[RuCl_5(OH_2)]$[11] were prepared according to literature procedures. Infrared spectra were recorded as CsI disks on a Mattson Galaxy Series FTIR 5000 spectrometer. NMR spectra were recorded on a Bruker Avance 300 MHz spectrometer with chemical shifts relative to Me_4Si (except for ^{15}N NMR, which are referenced to CD_3NO_2). EPR spectra were recorded on a Bruker EMX (Karlsruhe, Germany) spectrometer fitted with a high-sensitivity SP spherical cavity and a liquid-helium cryostat (Oxford Instruments, Eynsham, Witney U.K.). Electrospray ionization mass spectra were recorded on a Bruker-HP Esquire-LC ion trap mass spectrometer. Cyclic voltammograms were performed using a PAR model 273 potentiostat/galvanostat. Rate constants were determined using an Applied Photophysics DX17 stopped-flow spectrometer with a measured deadtime of 1 ms. Elemental analyses were carried out by Atlantic Microlabs Inc. (Norcross, GA). Inductively coupled plasma mass spectrometry (ICP-MS) measurements were recorded by Elemental Research Inc. (North Vancouver, BC, Canada).

Syntheses. AMD6245. K[Ru(Hedta)Cl]·$2H_2O$ (16.0 g, 32.0 mmol) was heated to reflux in deionized water (750 mL) for 2 h. The volume of the solution was reduced to half the original volume, and the solution was seeded with approximately 2−3 mg of Ru(Hedta)(OH_2). Upon cooling, a precipitate formed which was removed by filtration and washed with ice-cold water, ethanol, and diethyl ether. The product was dried in vacuo at 40 °C overnight (10.0 g, 77%). Anal. Calcd for $C_{10}H_{15}N_2O_9Ru$: C, 29.42; H, 3.70;N, 6.86; Cl, 0.0. Found: C, 29.34; H, 3.66; N, 6.92; Cl, 0.0. ES-MS: *m*/*z* 390, $[M - OH_2 - H]^-$. IR [CsI; ν (cm^{-1})]: 3148 (OH); 1741 (CO_2H); 1651 (CO_2^-).

AMD6263. AMD6245 (6.5 g, 16 mmol) was suspended in H_2SO_4 (0.1 M, 250 mL) and heated to reflux. Sodium nitrite (2.21 g, 32 mmol) dissolved in a minimum amount of water was added to the AMD6245 solution. The reaction mixture was heated at reflux for 30 min and was then removed from the heat and cooled. The solvent was removed, and the residue was stirred in water and filtered. The purple solid was washed with water, ethanol, and diethyl ether and dried in vacuo (5.76 g, 82%). ^{13}C NMR (D_2O): δ 182.73, 179.42, 178.72, 169.69, 66.54, 66.29, 65.30, 64.90, 63.44, 61.07. Anal. Calcd for $C_{10}H_{13}N_3O_9Ru·H_2O$: C, 27.40; H, 3.45; N, 9.59; Cl, 0. Found: C, 27.51; H, 3.51; N, 9.66; Cl, 0. ES-MS: *m*/*z* 420, $[M - H]^-$. IR [CsI; ν (cm^{-1})]: 3488 (H_2O); 1896 (NO); 1746 (CO_2H); 1684 (CO_2^-).

The analogous ^{15}N nitrosyl complex was prepared as above using enriched $Na^{15}NO_2$. IR [CsI; ν (cm^{-1})]: 3497 (H_2O); 1880 (NO); 1747 (CO_2H); 1674 (CO_2^-). ^{15}N NMR (D_2O): δ −14.83.

AMD6204. Ethylenediamine tetraacetic acid disodium salt (2.0 g, 5.0 mmol) was suspended in HCl (0.01 M, 20 mL). The salt dissolved when the suspension was heated to reflux. A solution of $K_2[RuCl_5(NO)]$ (2.0 g, 5 mmol) in HCl (0.01M, 20 mL) was added to the above ligand solution, and the reaction mixture was heated at reflux for 18 h. A beige/orange precipitate was removed by filtration, and the solvent was removed from the filtrate in vacuo. The residue was dissolved in deionized water and passed through a Dowex 50WX2 200 mesh H^+ ion exchange column. The brown band was collected in fractions, and each fraction was analyzed by UV−vis spectroscopy. The fractions containing identical spectra (λ_{max} = 521 nm; 376 nm) were combined and the solvent removed in vacuo to leave a brown-purple precipitate (1.06 g, 43.8%), which was dried in vacuo at room temperature. Anal. Calcd for $C_{10}H_{14}ClN_3O_9Ru·1.5H_2O$: C, 24.83; H, 3.54; N, 8.69; Cl, 7.33. Found: C, 24.93; H, 3.54; N, 8.71; Cl, 7.40. ES-MS: *m*/*z* 480, $[M + Na]^+$; *m*/*z* 458, $[M + H]^+$. IR [CsI; ν (cm^{-1})]: 1904 (NO); 1736 (CO_2H); 1663 (CO_2^-); 332 (Ru−Cl).

AMD6221. Diethylenetriaminepentaacetic acid (142.0 g, 360 mmol) was dissolved in HCl (1.0 mM, 1.0 L) by heating to reflux temperature. After 30 min the ligand was completely dissolved, at which time $K_2[RuCl_5(OH_2)]$ (135.0 g, 360 mmol) dissolved in a solution of HCl (1.0 mM, 1.2 L) was added in one portion. The solution was stirred with the aid of a mechanical stirrer. The reaction mixture was heated at reflux for 2 h (within 1 h the solution had turned yellow and a precipitate began to form). The yellow solution was filtered while hot, and the collected precipitate was washed with ice-cold water, ethanol, and finally diethyl ether. The yellow solid was dried in vacuo overnight (100.5 g, 51%). Anal. Calcd for $C_{14}H_{21}ClN_3O_{10}Ru·H_2O$: C, 30.80; H, 4.25; N, 7.70; Cl, 6.49. Found: C, 30.68; H, 4.34; N, 7.70; Cl, 6.49. ICP-MS Anal. Calcd for $C_{14}H_{21}ClN_3O_{10}Ru·H_2O$: K, 0; Ru, 18.52. Found: K, 0; Ru, 18.3. ES-MS: *m*/*z* 491, $[M - Cl - 2H]^-$. IR [CsI; ν (cm^{-1})]: 1726 (CO_2H); 1667 (CO_2^-).

AMD3689. AMD6221 (20.0 g, 36.6 mmol) was added to a nitrogen-purged solution of H_2SO_4 (0.1 M, 400 mL). The reaction mixture was stirred under a N_2 atmosphere with the aid of a mechanical stirrer. Sodium nitrite (10.1 g, 146.5 mmol) was added to the solution, and the reaction mixture was heated to reflux. After 20 min the solution turned a deep red color. After 2 h the reaction mixture was removed from heat and cooled. A light purple precipitate formed, which was removed by filtration and washed with ice-cold water, ethanol, and diethyl ether. The solid was then dried in vacuo at room temperature (9.9 g, 49%). ^{13}C NMR (D_2O/K_2CO_3): δ 182.30, 179.57, 174.28, 173.06, 171.69, 68.17, 67.07, 64.81, 63.01, 61.89, 61.07, 60.04, 59.96, 57.01. Anal. Calcd for $C_{14}H_{20}N_4O_{11}Ru·0.3H_2O$: C, 31.92; H, 3.94; N, 10.64; Cl, 0. Found: C, 31.96; H, 3.92; N, 10.68; Cl, 0. ES-MS: *m*/*z* 523, $[M + H]^+$. IR [CsI; ν (cm^{-1})]: 3450 (H_2O); 1913 (NO); 1730 (CO_2H); 1680 (CO_2^-).

The analogous ^{15}N nitrosyl complex was prepared as above using enriched $Na^{15}NO_2$. IR [CsI; ν (cm^{-1})]: 3453 (H_2O); 1885 (NO); 1738 (CO_2H); 1684 (CO_2^-); 1638 (CO_2^-). ^{15}N NMR (D_2O): δ −14.17.

X-ray Diffraction Data Collection and Solution and Refinement for AMD6245, AMD6263, and AMD3689. Crystallographic data and refinement parameters are given in Table 6 for AMD6245, Table 7 for AMD6263, and Table 8 for AMD3689. Intensity data were measured on a Bruker SMART system with Mo Kα radiation at 293 K for AMD6245 and AMD6263 and at 94 K for AMD3689. Data collection and reduction were performed using the SAINT processing program. Direct methods solution and refinements (full-matrix least squares on F^2) were performed using the SHELTXL program. Yellow crystals of AMD6245 were obtained from slow cooling and evaporation of the reaction mixture.

Purple crystals of AMD6263 suitable for X-ray crystallography studies were grown from an aqueous solution of the complex upon slow evaporation.

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Table 6. Details of Data Collection and Structure Refinement for AMD6245

chem formula	$C_{10}H_{14}N_2O_9Ru$	fw	407.30
a	8.4382(2) Å	space group	$P2_1/c$
b	8.8304(2) Å	T	293(2) K
c	17.6321(4) Å	λ	0.710 73 Å
α	90°	D_{calcd}	2.089 Mg/m^3
β	99.603°	μ(Mo Kα)	1.265 mm^{-1}
γ	90°	R1[a]	0.0559
V	1295.3 (2) Å^3	wR2[b]	0.1094
Z	4		

[a] $R1 = \sum(F_o - F_c)/F_o$. [b] $wR2 = [\sum(F_o^2 - F_c^2)^2/\sum wF_o^2]^{1/2}$.

Table 7. Details of Data Collection and Structure Refinement for AMD6263

chem formula	$C_{10}H_{14}N_3O_{10}Ru$	fw	437.31
a	9.9043(4) Å	space group	$P2_1/c$
b	13.1144(3) Å	T	293(2) K
c	12.0914(4) Å	λ	0.710 73 Å
α	90°	D_{calcd}	1.879 Mg/m^3
β	100.191°	μ(Mo Kα)	1.073 mm^{-1}
γ	90°	R1[a]	0.0525
V	1545.8 (5) Å^3	wR2[b]	0.1203
Z	4		

[a] $R1 = \sum(F_o - F_c)/F_o$. [b] $wR2 = [\sum(F_o^2 - F_c^2)^2/\sum wF_o^2]^{1/2}$.

Table 8. Details of Data Collection and Structure Refinement for AMD3689

chem formula	$C_{14}H_{24.56}N_4O_{13.28}Ru$	fw	562.49
a	8.838(2) Å	space group	$P\bar{1}$
b	9.452(3) Å	T	94 K
c	13.419(4) Å	λ	0.71073 Å
α	78.413 (6)°	D_{calcd}	1.814 Mg/m^3
β	75.804(6)°	μ(Mo Kα)	0.843 mm^{-1}
γ	73.562(6)°	R1[a]	0.0385
V	1031.8(5) Å^3	wR2[b]	0.1060
Z	2		

a $R1 = \sum(F_o - F_c)/F_o$. [b] $wR2 = [\sum(F_o^2 - F_c^2)^2/\sum wF_o^2]^{1/2}$.

Purple crystals of AMD3689 suitable for X-ray crystallography were grown upon slow evaporation of an aqueous solution. The discrepancy between the analytical data, which supports the formulation $C_{14}H_{20}N_4O_{11}Ru\cdot 0.3H_2O$, and the formulation $C_{14}H_{20}N_4O_{11}Ru\cdot 2.28H_2O$ reflects the difference in sample handling. Crystals used in the structural determination were mounted in the mother liquor and immediately frozen in a stream of liquid nitrogen. In contrast, the sample used for elemental analysis was dried at room temperature, a procedure that results in a loss of crystallinity as a consequence of loss of water of crystallization. This observation was confirmed by TGA of a freshly recrystallized sample which exhibited a weight loss of ca. 7.5% (theoretical based on $C_{14}H_{20}N_4O_{11}Ru\cdot 2.28H_2O$: 7.30%).

EPR Measurements. The EPR spectra were recorded using the following conditions: $T = 10$ K; microwave frequency = 9.470 GHz; microwave power = 50.41 μW; time constant = 81.92 ms; modulation amplitude = 5 G. Solutions of AMD1226 or AMD6221 (100 μM) were prepared in sodium phosphate (50 mM) buffered to pH = 7.4. The sample was frozen in liquid nitrogen and the EPR spectrum recorded, relative to dpph as an external standard. The sample was warmed to room temperature and NO gas was introduced via syringe and bubbled through the solution for 30 s. The sample was frozen again in liquid nitrogen and the EPR spectrum measured.

Electrochemistry. Electrochemical measurements were carried out under conditions reported previously[17] with a Ag/AgCl reference electrode and a Pt wire working electrode. Solutions of ruthenium complex (2 mM) were prepared in sodium acetate (50 mM) buffered to pH = 5.5. Solutions were degassed by purging with argon for 15 min and then kept under an argon atmosphere. Solutions were scanned first to negative potentials from 0 or 0.1 V to −0.45 V and back to 0 V. Scans toward positive potentials were then performed from 0 to 1.2 V and back to 0 V. All scans were performed at a rate of 100 mV/s.

Kinetic Studies. In the stopped flow experiment the temperature was maintained within ±0.1 °C. One syringe was charged with a saturated solution of NO (see below) and one with the ruthenium complex AMD1226 (50 μM) or AMD6221 (100 μM) so that the final concentration of complex was 25 or 50 μM after mixing. Both the NO and complex solution were prepared in potassium phosphate buffer (50 mM) at pH = 7.4. The calculated rate constants are determined from an average of at least 6 experiments.

The NO solution was prepared as follows: Sulfuric acid (2 M) was dropped onto solid sodium nitrite under a nitrogen atmosphere in a Kipps apparatus. The gas produced was then purged by passing through a NaOH (1 M) solution, distilled H_2O, and finally through a cold trap to remove impurities. A degassed potassium phosphate buffer solution was placed under an atmosphere of NO accompanied by agitation to ensure saturation. The concentration of the NO solution was quantified by a titration with deoxyferrous myoglobin as described in the literature.[1,18]

RAW264 Murine Macrophage Assay for NO Scavenging by Ruthenium Complexes. RAW264 cells were cultured on 24 well plates (2×10^6 cells/well) in 2 mL of Eagle's minimal essential medium. The cells were activated by the addition of 10 μg/mL *Escherichia coli* 0111:B4 lipopolysaccharide (Sigma L2630) and 100 IU/mL mouse recombinant IFN-γ. The production of nitric oxide was estimated from the amount of nitrite in the medium after 18 h using the Greiss assay.[21] To estimate the NO scavenging ability of the ruthenium complexes, the nitrite accumulation was measured under the following conditions: (1) LPS/IFN-γ activated cells; (2) LPS/IFN-γ activated cells treated with appropriate amount of Ru complex. The cells were activated to produce NO in the presence of the appropriate ruthenium complex (100 μM), and the results are reported as the change in the amount of nitrite produced between treated cells and nontreated cells. A dose response of NO scavenging by AMD6221 was determined by adding varying amounts (12.5−200 μM) of the complex to the cells. Control experiments were performed to show that ruthenium complexes were not cytotoxic at the concentrations used in this study, as determined from an MTT assay.[21]

Supporting Information Available: An X-ray crystallographic file in CIF format for the structures of AMD6245, AMD6263, and AMD3689 and figures for EPR, CV, and electrochemical plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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