Characterization by NMR Spectroscopy, X-ray Analysis and Cytotoxic Activity of the Ruthenium(II) Compounds $[RuL_3](PF_6)_2$ (L = 2-Phenylazopyridine or *o*-Tolylazopyridine) and $[RuL'_2L'']](PF_6)_2$ (L′, L′′ = 2-Phenylazopyridine, 2,2′-Bipyridine)

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Tris(ligand) complexes $[RuL_3](PF_6)_2$ (L = 2-phenylazopyridine or *o*-tolylazopyridine) and mixed ligand $[RuL'_2L'']](PF_6)_2$ (L′ and L′′ are 2-phenylazopyridine or 2,2′-bipyridine) have been synthesized, structurally characterized and investigated for cytotoxic activity. These complexes are important to study the hypothesis that the compound α-$[Ru(azpy)_2Cl_2]$ (azpy = 2-phenylazopyridine) exhibits a high cytotoxicity due to its two *cis* chloride ligands, which might be exchanged for biological targets as DNA. Molecular structures of *mer*-$[Ru(azpy)_3](PF_6)_2$ (**1**) and *mer*-$[Ru(tazpy)_3](PF_6)_2$ (**5**) (tazpy = *o*-tolylazopyridine) have been determined by X-ray diffraction. Series of complexes $[RuL_3](PF_6)_2$ and $[RuL'_2L'']](PF_6)_2$ show interesting NMR spectroscopic data; e.g. the spectrum of *mer*-$[Ru(azpy)_3](PF_6)_2$ (**1**) shows extremely broadened resonances at room temp. but sharpened resonances at low temperature. In the ^1H NMR spectra of compounds $[Ru(azpy)_2(bpy)]^{2+}$ and $[Ru(bpy)_2(azpy)]^{2+}$ (bpy = 2,2-bipyridine), respectively, less broadened (room temp.) or completely sharp resonances (room temp.) occur in comparison to **1** (under same conditions). By selecting the right temperature and/or concentration, NMR spectra of these series of compounds have been resolved using 2D COSY and NOESY NMR spectroscopy. Remarkably, the cytotoxicity data against a series of human tumor cell lines (A498, EVSA-T, H226, IGROV, M19, MCF-7 and WIDR) show a moderate cytotoxicity for these series of tris(ligand) complexes. So, even though no chloride ligands are present in these tris(ligand) complexes, a considerable cytotoxic activity is observed. This would imply that the 2-phenylazopyridine ruthenium(II) complexes act by a completely different mechanism than the well-known cisplatin. This finding is important, because an anticancer compound acting via a different mechanism is a prerequisite in designing new anticancer drugs.

Introduction

The dichlorobis(2-phenylazopyridine)ruthenium(II) complexes, $[Ru(azpy)_2Cl_2]$ are under renewed investigation due to their cytotoxic activity in a panel of human tumor cell lines.[1,2] In particular the α configuration (Figure 1, α indicating the pairs of coordinating Cl, Npy and Nazo atoms in a mutual *cis*, *trans*, *cis* orientation) of the $[Ru(azpy)_2Cl_2]$ complexes is the most promising isomer, as this isomer displays a very high cytotoxicity, stability and a reasonable solubility.[1,2] The mechanisms underlying the cytotoxicity of the $[Ru(azpy)_2Cl_2]$ complexes and in particular of the α-$[Ru(azpy)_2Cl_2]$ are not known yet. In analogy to cisplatin[3] it has been hypothesized that two labile *cis* chloride ligands contribute to the overall activity of the compound, as in the tumor cell these labile chloride ligands will be replaced by water ligands and subsequently replaced by the nucleotides of the DNA. To confirm or reject this hypothesis it was decided to replace the chloride ligands of the *cis*-$[Ru(azpy)_2Cl_2]$ complexes by a third azpy or bpy ligand, unable to dissociate, and investigate this series tris(ligand)rutheni-

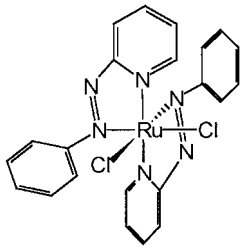

Figure 1. Schematic representation of the compound α-$[Ru(azpy)_2Cl_2]$.

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um(II) compounds for their cytotoxicity. This will result in a series of compounds, which will give more insights in the structure-activity relationships of these type of complexes.

Perhaps the most frequently considered binding interaction of heavy metals with DNA is that of coordinative binding to the bases of the DNA.[4] In addition to this interaction, intercalation is a common mode of association of small molecules with DNA; a flat aromatic heterocyclic moiety inserts and stacks in between the DNA base pairs.[4] Already in 1974 Lippard and co-workers[5,6] determined that platinum(II) complexes containing an aromatic heterocyclic ligand, such as terpyridine, could intercalate in DNA. This intercalation is not restricted to completely flat square-planar complexes, but partial intercalation of ligands coordinated to octahedral metal centers is also possible.[7] A ruthenium compound very thoroughly studied for its interaction with DNA is the tris(phenanthroline)ruthenium(II) complex. Depending on its enantiomeric form it binds to DNA by two non-coordinative modes, one described as a surface- or groove-bound interaction in the minor groove of the helix and the other appearing to be an intercalative interaction in the major groove of the helix.[4,7–9] However, the exact DNA binding mode of even a simple species like $[Ru(phen)_3]^{2+}$ remains an issue of debate.[10]

The compound $[Ru(azpy)_3](PF_6)_2$ theoretically exists in two different isomeric forms disregarding ΔΛ enantiomerism, i.e. the *mer* and *fac* isomers (Figure 2). The symmetry properties of the two isomers are markedly different; *fac*-$[Ru(azpy)_3](PF_6)_2$ belongs to the C_3 point group and hence has three equivalent azpy ligands. The *mer* isomer on the other hand is of C_1 symmetry, and all azpy ligands are inequivalent. The *fac* isomer formation of $[Ru(azpy)_3]^{2+}$ appears unlikely, also because previous studies have reported[11,12] that the *fac* isomer of $[Ru(azpy)_3]^{2+}$ is sterically unfavorable due to crowding of the three phenyl groups.

Several papers report the synthesis of $[Ru(azpy)_3]^{2+}$-like complexes. The first described[13] synthesis of $[Ru(azpy)_3]^{2+}$ dates from 1982; $[Ru(azpy)_3](PF_6)_2$ was synthesized using azpy and a crude mixture of isomers of $[Ru(azpy)_2Cl_2]$ with one isomer (probably α) in excess. The product is described as "a mixture" which has been characterized by IR and UV/Vis, which unfortunately does not say anything about the possible obtained *mer* or *fac* isomer(s). A one-pot synthesis using $RuCl_3$ and $[Ag(azpy)_2]^+$ as synthon has also been reported, but in this paper the possibility of the formation of both *fac* and *mer* isomers was not even discussed.[14] A recent publication appeared[11] after the present experimental work was carried out and showed the crystal structure of *mer*-$[Ru(azpy)_3](ClO_4)_2$. Except this crystal structure only a few more crystal structures of the type $[M(azpy)_3]^{n+}$ (with M any metal) have been reported, i.e. the rhenium structure[15] *fac*-$[Re(azpy)_3](ClO_4)$, the iron compound[16] $[Fe_2(CO)_2(4\text{-}CH_3\text{-}2\text{-phenylazopyridine})_3]$, the compound[11] *mer*-$[Ru(HL)_3](ClO_4)_2$ {HL = 2-[(4-(methoxyamino)phenyl)azopyridine} and the rhenium compound[17] *fac*-$[Re(apy)_3]I$ (apy = 2,2′-azobipyridine). Another type of tris(ligand) complexes are the mixed ligand complexes of the type $RuLL'_2$ (with L and L′ both didentate ligands with one of them containing the azo functionality) of which the crystal structures of $[Ru(bpy)(MeaaiMe)_2](ClO_4)_2$ [MeaaiMe = 1-methyl-2-(*p*-methylazo)imidazole], $[Ru(bpy)_2(p\text{-}MeaaiCH_2ph)](ClO_4)_2\cdot H_2O$, $[Ru(phen)(HaaiMe)_2](ClO_4)_2$, $[Ru(azpy)_2(H_2biim)](ClO_4)_2\cdot 3CH_2Cl_2$ (H_2Biim = 2,2′-biimidazole) and $[Ru(HL)(bpy)_2](ClO_4)_2$ {HL = 2-[(4-(arylamino)phenyl)azopyridine} are nice examples[11,18–21] of compounds structurally related to the series of compounds

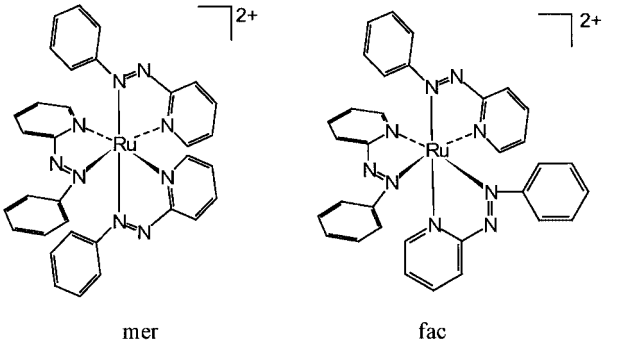

Figure 2. *mer*- and *fac*-$[Ru(azpy)_3]^{2+}$ (only Δ isomers shown).

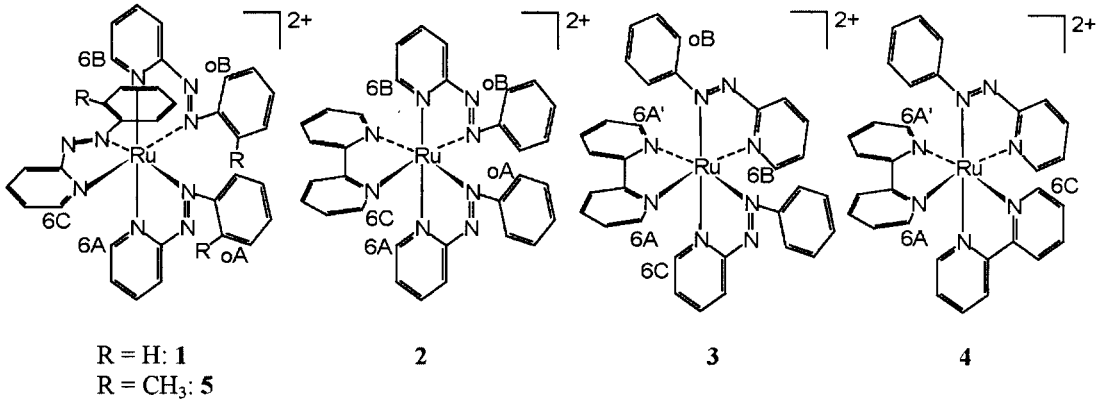

Figure 3. A series of tris(ligand) ruthenium(II) complexes (from left to right): mer-$[Ru(azpy)_3](PF_6)_2$ (**1**) and *mer*-$[Ru(tazpy)_3](PF_6)_2$ (**5**), α-$[Ru(azpy)_2(bpy)](PF_6)_2$ (**2**), β-$[Ru(azpy)_2(bpy)](PF_6)_2$ (**3**) and $[Ru(azpy)(bpy)_2](PF_6)_2$ (**4**); some hydrogen atoms, important for NMR assignments, are indicated.

described in this paper. Till now, no 2D NMR characterization or cytotoxicity tests have ever been reported on any kind of above mentioned tris(ligand) complexes.

To design new anticancer drugs and to develop structure-activity relationships for 2-phenylazopyridine ruthenium(II) complexes, a series (Figure 3) of newly synthesized tris(ligand) ruthenium(II) complexes, i.e. *mer*-[Ru(azpy)$_3$](PF$_6$)$_2$ (**1**), α-[Ru(azpy)$_2$(bpy)](PF$_6$)$_2$ (**2**), β-[Ru(azpy)$_2$(bpy)](PF$_6$)$_2$ (**3**), [Ru(azpy)(bpy)$_2$](PF$_6$)$_2$ (**4**) and *mer*-[Ru(tazpy)$_3$](PF$_6$)$_2$ (**5**, tazpy = *o*-tolylazopyridine) are synthesized and characterized by 2D NMR spectroscopy. X-ray diffraction analysis of **1** and **5** have been performed. The synthesis of these complexes is known from early work,[12,13,22] but the full NMR characterization using variable temperature NMR and 2D NMR techniques and especially the correlations made to antitumor research are new. The cytotoxicity of the complexes will be discussed in relation to the very cytotoxic dichlorobis(azpy)ruthenium(II) complexes.

Results and Discussion

General Considerations

One of the syntheses described in this paper is the synthesis of *mer*-[Ru(azpy)$_3$](PF$_6$)$_2$ from α-[Ru(azpy)$_2$(NO$_3$)$_2$] and azpy. Since only the *mer* isomer of [Ru(azpy)$_3$](PF$_6$)$_2$ has two azpy ligands positioned according to the α configuration (pairs of pyridine N atoms *trans* and Nazo atoms *cis*) this synthetic route theoretically excludes *fac* isomer formation, assuming that no isomerization[23] takes place. Moreover, *fac* isomer formation remains unlikely, also because previous studies have reported[11,12] that the *fac* isomer of [Ru(azpy)$_3$](PF$_6$)$_2$ is sterically unfavorable due to crowding of the three phenyl groups (vide infra). As anticipated only pure *mer*-[Ru(azpy)$_3$](PF$_6$)$_2$ was obtained.

Crystal Structures of 1 and 5

The cations of the crystal structures of *mer*-[Ru(azpy)$_3$](PF$_6$)$_2$ (**1**) and *mer*-[Ru(tazpy)$_3$](PF$_6$)$_2$ (**5**) are redrawn in Figure 4. For clarity, only the Λ enantiomers are shown, while the unit cell actually contains the Λ and Δ enantiomer. Furthermore the two PF_6^- counterions of which one is disordered have been omitted. The X-ray data of *mer*-[Ru(azpy)$_3$](ClO$_4$)$_2$ have been published by Das et al,[11] but has a different crystal system (monoclinic) and space group ($P2_1/c$) than **1** (resp. triclinic and $P\bar{1}$). However, the bond lengths of the cation of *mer*-[Ru(azpy)$_3$](ClO$_4$)$_2$ show a great deal of similarity with **1**. It is interesting to compare the X-ray data of **1** with *mer*-[Ru(tazpy)$_3$](PF$_6$)$_2$ (**5**) (see Figure 4). Crystallographic data of **1** and **5** are listed in Table 1. Selected bond lengths and angles of **1** and **5** are listed in Table 2 and Table 3, respectively.

In order to draw a comparison of the tris(ligand) complexes **1** and **5** with the related bis(azpy) complexes, like α-[Ru(azpy)$_2$Cl$_2$], α-[Ru(azpy)$_2$(NO$_3$)$_2$] and β-[Ru(azpy)$_2$Cl$_2$] (β indicating coordinating pairs Cl, Npy, Nazo in mutual *cis*, *cis cis* orientation), some distances within these complexes are listed in Table 4. In the crystal structures[24,25] of the bis(azpy) complexes the Ru–Nazo bonds were found to be significantly shorter than the Ru–Npy bonds. These short Ru–Nazo bonds can be attributed to substantial π-back bonding of the ruthenium through an azo nitrogen [Ru(t_{2g})-π*azpy]. In complexes **1** and **5** the lengths of the Ru–Nazo bonds are, on average, more similar to the Ru–Npy bond lengths. Therefore, no significant π-back bonding effects on the coordination bonds of **1** and **5** are visible. This is probably caused by a greater competition for Ru(t_{2g}) electrons in the tris(ligand) complexes. A *trans* N(azo)–M–N(azo) arrangement is quite rare as can be directly seen from the fact that from the five isomers of [Ru(azpy)$_2$Cl$_2$] the less common isomers, i.e.[26] δ-[Ru(azpy)$_2$Cl$_2$] and the

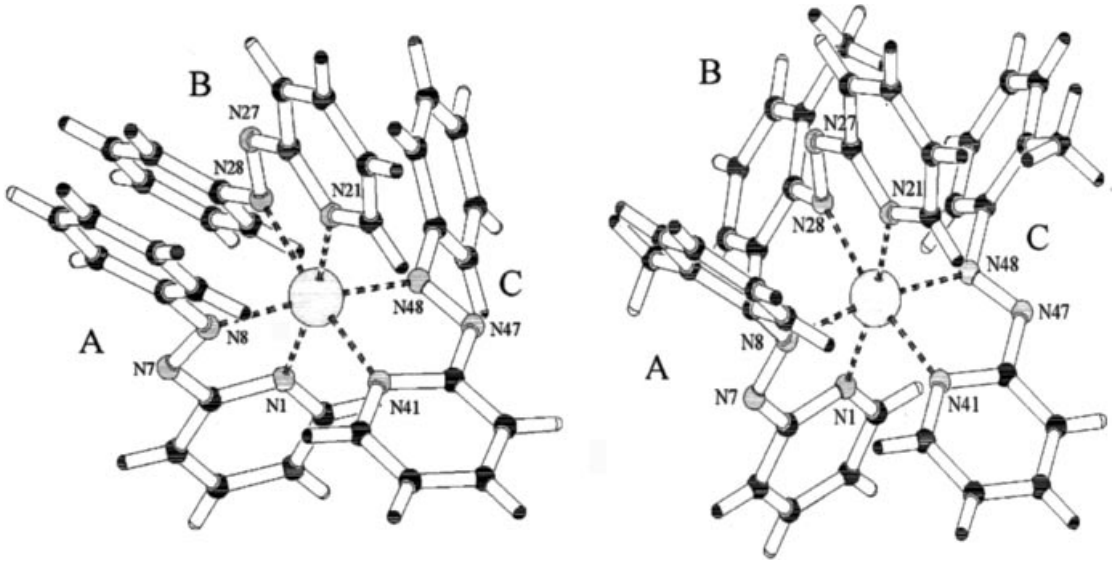

Figure 4. Crystal structures of **1** (left) and **5** (right). Only the Λ isomers are shown. To facilitate discussion the three (t)azpy ligands are denoted with A, B and C.

Table 1. Crystallographic data for crystal structure determinations of **1** and **5**.

Compound	**1**	**5**
Empirical formula	$C_{33}H_{27}N_9Ru{\cdot}2PF_6$	$C_{36}H_{33}N_9Ru{\cdot}2PF_6$
Formula mass	940.65	982.72
Crystal system	triclinic	triclinic
Space group	$P\bar{1}$ (No. 2)	$P\bar{1}$ (No. 2)
a [Å]	9.4164(12)	10.8271(10)
b [Å]	10.1664(12)	11.9490(10)
c [Å]	19.230(3)	15.866(2)
α [°]	86.606(10)	95.902(6)
β [°]	88.751(10)	99.356(6)
γ [°]	88.105(10)	93.930(9)
V [Å]^3	1836.3(4)	2007.0(4)
D_c [g cm^{-3}]	1.701	1.626
Z	2	2
μ (Mo-K_α) [mm^{-1}]	0.615	0.567
Crystal color	dark red	black
Crystal size [mm]	$0.1\times0.3\times0.3$	$0.10\times0.25\times0.35$
Total data	23859	43393
Total unique data	6474	9124
R_{int}	0.035	0.0455
No. of refined parameters	537	544
R_1[a] [$I > 2\sigma(I)$]	0.0312 [5629 refl.]	0.0271 [7824 refl.]
wR_2[b] (all data)	0.0732	0.0631
GoF	1.074	1.032

[a] $R_1 = \Sigma||F_o| - |F_c||/\Sigma|F_o|$. [b] $wR_2 = [\Sigma\,[w(F_o^2 - F_c^2)^2]/\Sigma\,[w(F_o^2)^2]]^{1/2}$.

Table 2. Selected bond lengths [Å] and angles [°] of **1**.

Ru(1)–N(1)	2.067(2)	N(1)–Ru(1)–N(28)	99.69(8)
Ru(1)–N(8)	2.0776(19)	N(1)–Ru(1)–N(41)	90.45(8)
Ru(1)–N(21)	2.046(2)	N(1)–Ru(1)–N(48)	95.65(8)
Ru(1)–N(28)	2.049(2)	N(8)–Ru(1)–N(21)	96.19(8)
Ru(1)–N(41)	2.053(2)	N(8)–Ru(1)–N(28)	80.30(8)
Ru(1)–N(48)	2.0216(19)	N(8)–Ru(1)–N(41)	103.65(8)
N(7)–N(8)	1.278(3)	N(8)–Ru(1)–N(48)	170.94(8)
N(27)–N(28)	1.281(3)	N(21)–Ru(1)–N(28)	76.16(8)
N(47)–N(48)	1.282(3)	N(21)–Ru(1)–N(41)	93.95(8)
		N(21)–Ru(1)–N(48)	92.86(8)
		N(28)–Ru(1)–N(41)	169.79(8)
N(1)–Ru(1)–N(8)	75.30(8)	N(28)–Ru(1)–N(48)	101.91(8)
N(1)–Ru(1)–N(21)	171.16(8)	N(41)–Ru(1)–N(48)	75.68(8)

Table 3. Selected bond lengths [Å] and angles [°] of **5**.

Ru(1)–N(1)	2.0651(15)	N(1)–Ru(1)–N(28)	102.28(6)
Ru(1)–N(8)	2.0278(14)	N(1)–Ru(1)–N(41)	85.51(6)
Ru(1)–N(21)	2.0500(15)	N(1)–Ru(1)–N(48)	100.02(6)
Ru(1)–N(28)	2.0695(15)	N(8)–Ru(1)–N(21)	95.11(6)
Ru(1)–N(41)	2.0665(14)	N(8)–Ru(1)–N(28)	90.04(6)
Ru(1)–N(48)	2.0379(14)	N(8)–Ru(1)–N(41)	95.57(6)
N(7)–N(8)	1.282(2)	N(8)–Ru(1)–N(48)	171.73(6)
N(27)–N(28)	1.285(2)	N(21)–Ru(1)–N(28)	76.18(6)
N(47)–N(48)	1.280(2)	N(21)–Ru(1)–N(41)	96.85(6)
		N(21)–Ru(1)–N(48)	88.88(6)
		N(28)–Ru(1)–N(41)	170.84(6)
N(1)–Ru(1)–N(8)	76.08(6)	N(28)–Ru(1)–N(48)	97.95(6)
N(1)–Ru(1)–N(21)	171.11(6)	N(41)–Ru(1)–N(48)	75.73(6)

probable existing[27] ε-$[Ru(azpy)_2Cl_2]$ (δ indicating Cl, Npy and Nazo, *trans*, *trans*, *trans* and ε has the pairs of Cl, Npy and Nazo *cis*, *cis*, *trans*) have this *trans* Nazo-M–Nazo grouping. Also in the meridional tris(ligand) complexes this *trans* N(azo)–M–N(azo) arrangement occurs, e.g. the iron compound[16] $[Fe_2(CO)_2(4\text{-}CH_3\text{-}2\text{-phenylazopyridine})_3]$ and the compound[11] *mer*-$[Ru(azpy)_3](ClO_4)_2$. Especially in the *trans* Nazo–M–Nazo arrangement the two azo bonds are thought to compete for the back bonding of the Ru^{II} (4d) t2g electron density, resulting in, in theory, relatively long Ru–Nazo bond lengths.

Table 4. Comparison of selected bond lengths [Å] from the crystallographic data[24,25] of α-$[Ru(azpy)_2Cl_2]$, α-$[Ru(azpy)_2(NO_3)_2]$, β-$Ru(azpy)_2Cl_2]$, **1** and **5**. Distances are reported in Å, with standard uncertainties in parentheses. β-$Ru(azpy)_2Cl_2$ has two independent molecules.

	Ru–Naza	Ru–Npy	Naza-Naza
α-$Ru(azpy)_2Cl_2]$	1.977(4)	2.045(4)	1.279(7)
	1.984(4)	2.051(4)	1.283(6)
α-$Ru(azpy)_2(NO_3)_2]$	2.014(4)	2.031(4)	1.282(5)
	1.960(4)	2.059(4)	1.270(5)
β-$Ru(azpy)_2Cl_2]$ (mol, 1)	1.960(9)	2.059(9)	1.30(1)
	2.003(9)	2.020(9)	1.29(1)
β-$Ru(azpy)_2Cl_2]$ (mol, 2)	1.958(9)	2.069(9)	1.31(1)
	1.96(1)	2.028(9)	1.30(2)
1	2.0776(19)	2.067(2)	1.278(3)
	2.049(2)	2.046(2)	1.281(3)
	2.0216(19)	2.053(2)	1.282(3)
5	2.0278(14)	2.0651(15)	1.282(2)
	2.0695(15)	2.0500(15	1.285(2)
	2.0379(14)	2.0665(14)	1.280(2)

^{1}H NMR Characterization of the Tris(ligand)ruthenium(II) Complexes

General Information

At room temperature, the resonances of the tris(ligand) complexes **1**,**2** and **5** were found to be significantly broadened at room temperature. The variable-temperature ^{1}H NMR spectra in $[D_6]$acetone of **1** and **2** are depicted in Figure 5. This broadening seems to be dependent on both concentration and temperature.

For example compound **1** shows really broad resonances at room temperature but cooling the sample results in marked sharpening of the resonances (Figure 5). Till now, no thorough NMR characterization has been reported about *mer*-$[Ru(azpy)_3]^{2+}$ type of compounds. For example the reported ^{1}H NMR spectrum of the compound[12] *mer*-$[Ru(tap)_3]^{2+}$ (tap = *m*-tolylazopyridine) shows two methyl resonances and the absence of one more methyl signal (as all three ligands in the *mer* isomer should be distinguishable) has been explained by the fact that in practice two rings become equivalent. By using pure β-$[Ru(tap)_2(H_2O)_2]^{2+}$ the occurrence of both *mer* and (a small amount of) *fac*-$[Ru(tap)_3]^{2+}$ have been reported which have been characterized by ^{1}H NMR spectroscopy.[12] However, this NMR characterization was not very clear. Only the high field region of the ^{1}H NMR spectrum was shown, which is quite inconvenient with respect to the broadening of especially the aromatic resonances observed for this type of complexes (vide infra).[12] Also the recent publication of Das et al. does not show any NMR spectroscopic data of *mer*-$[Ru(azpy)_3]$-

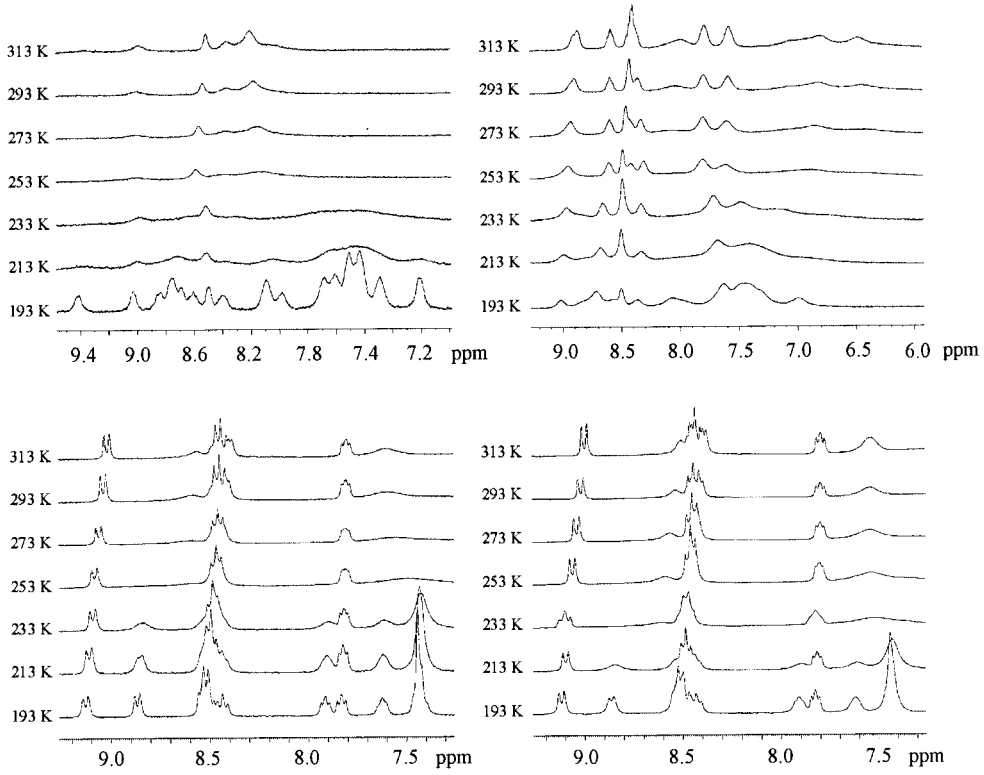

Figure 5. Temperature-dependent ^{1}H NMR spectra (300 MHz) of **1** (upper spectra) and **2** (lower spectra) at 2 mM (left) and 6 mM (right) in [D_6]acetone.

$(ClO_4)_2$. In general, for tris(ligand) complexes with 2-phenylazopyridine- or 2-phenylazoimidazole-like ligands only ^{1}H NMR chemical shift data are mentioned,[19–21,28] without performing 2D NMR experiments and no broadening is mentioned. In our opinion the ^{1}H NMR spectroscopic data of these very complicated spectra[19–21,28] are questionable. Therefore, an extensive description of the assignment of the tris(ligand) complexes mentioned in this paper using several 2D NMR techniques seems to be useful.

The ^{1}H NMR chemical shift values (600 MHz) of the complexes **1**, **2**, **3** and **4** (samples of **1** and **2** recorded at low temperatures) are listed in Table 5 (the resonances belonging to the azpy ligands are denoted with A, B or C, e.g. 6A or *o*B; the resonances belonging to the bpy ligands are denoted by only a number). The assignment of *mer*-[Ru(tazpy)$_3$](PF$_6$)$_2$ has not been attempted, as the broadening in this case was even more complicated probably due to additional steric hindrance due to the methyl group of the phenyl rings and under no conditions sharp resonances were observed. The assignment of the resonances of **1** at low temperature will be described in detail to show the common way of assigning such complicated spectra. It

Table 5. ^{1}H NMR chemical shift values (600 MHz) of **1**, **2**, **3** and **4** in [D_6]acetone. The spectrum of **1** has been recorded recorded at 183 K, 2 at 195 K and **3** and **4** at room temperature.

Compound (ligand)	azpy bpy 6 6	5 5	4 4	3 3	*o* 6′	*m* 5′	*p* 4′	3′
1 (A)	8.70	7.99	8.40	8.78	7.53	7.44	7.68	
(B)	8.51	8.10	8.62	8.87	7.02	7.29	7.50	
(C)	9.06	8.08	8.75	9.44	7.42	7.61	7.71	
2 (A = B)	8.55	7.92	8.44	8.88	7.43	7.45	7.63	
(bpy)	8.53	7.83	8.50	9.14				
3 (bpy)	8.29	7.70	8.17	8.47	7.63	7.82	8.43	8.67
(B)*	8.26	7.91	8.65	9.21	7.80	7.15	7.39	
(C)*	8.21	8.04	8.69	9.15	7.36	7.59	7.70	
4 (bpyA)	8.05	8.13	7.56	8.51	7.72	8.37	7.73	8.65
(bpyC)	8.39	8.39	7.75	8.96	8.02	8.39	7.70	8.99
(azpyB)	8.29	8.44	7.84	9.02	7.18	7.23	7.43	

*pyridine ring B and C cannot be distinguished.

makes in a beautiful way use of the fact that in the compound *mer*-$[Ru(azpy)_3](PF_6)_2$, the azpy ligands are two-by-two in mutual α, β and ε configurations as shown in the bis(chelate) complexes $[Ru(azpy)_2Cl_2]$ and subsequently these configurations have known[26] interligand NOE cross-peaks. The use of these known geometries of $[Ru(azpy)_2Cl_2]$ complexes plays a key role in the assignment of compound **1.**

NMR Characterization of 1

The ^1H NMR spectrum of **1** (600 MHz) at low temperature and low concentration shows 16 well-resolved resonances some of which appear to be multiplet signals due to overlap of resonances (Figure S1, supporting information, for supporting information see also the footnote on the first page of this article). As **1** has C_1 symmetry, all three azpy ligands are inequivalent, resulting in 21 resonances, which have been fully assigned using 2D COSY and NOESY NMR spectroscopy.

First, from 2D COSY NMR the three sets of azpy pyridine signals and three sets of azpy phenyl signals have been distinguished. As in the NMR spectra only three sets of phenyl signals appear, the phenyl rings are assumed to rotate fast on the NMR time scale. The H6 protons of the azpy ligands were easily recognised from the smaller *J* coupling in comparison to the J coupling of the H3 atoms and appear at δ = 8.51, 8.70 and 9.06 ppm. The NOESY spectrum (Figure 6) shows several NOE cross peaks (Table S1, Supporting Information), i.e. one *o*-*o* coupling, one coupling between two H6 hydrogen atoms and four cross peaks between H6 and *ortho* resonances. The *mer*-$[Ru(azpy)_3]^{2+}$ backbone consists of pairs of azpy ligands of the isomeric bis(ligand) complexes, α-$Ru(azpy)_2$ (in Figure 4 these have been denoted as azpy ligand A and B), β-$Ru(azpy)_2$ (in Figure 4 these have been denoted as B and C) and ε-$Ru(azpy)_2$ (azpy ligands C and A in Figure 4). So because the azpy ligands A and B of **1** actually represent the α configuration, A6-*o*B and B6-*o*A NOEs are expected[24,26] in the 2D NOESY spectrum of **1**. Likewise, ligands B and C in **1** are expected[26] to display B6–C6 and *o*B-*o*C NOEs. The synthesis of the ε isomer, ε-$[Ru(azpy)_2Cl_2]$ has been reported in a short letter,[27] but no NMR spectroscopic data have been presented. Therefore the crystal structure of **1** is used to determine which NOE cross peaks are to be expected for this ε configuration. According to distances in the crystal structure of **1** the unknown ε-$Ru(azpy)_2$ backbone should give rise to 6C-*o*A (average distance of H46–H10 and H44–H14 = 4.0 Å) and 6A-*o*C cross peaks (average distance of H6–H47 and H6–H54 = 3.7 Å).

A close look in the NOESY spectrum shows that one of the H6 resonances (at, 8.70 ppm) gives cross peaks to two *ortho* signals. These NOEs unambiguously assign the resonance at δ = 8.70 ppm to correspond to the H6A atom giving cross peaks to *o*B and *o*C. In fact, the NOE 6A-*o*B is expected, as this is the known cross peak for the α configuration. The 6A-*o*C is a cross peak apparently occurring in the ε configuration, as was also likely from the crystal structure. How to discriminate between the *o*B and *o*C resonances? One of these *ortho* resonances is shifted upfield related to the other. The *o*B hydrogens are positioned within the shielding cone of pyridine ring A. For this reason the high-field resonance at δ = 7.02 ppm can be assigned to the *o*B hydrogens. A cross peak occurs between *o*B and *o*C in agreement with the β configuration. Two H6 resonances remain: the H6 resonances at δ = 8.51 and 9.06 ppm, which present a cross peak between each other. This 6B-6C NOE perfectly agrees with the β-$Ru(azpy)_2$ configuration. The signal at δ = 8.51 ppm is assigned to 6B, as this resonance is within the shielding cone of the pyridine ring of ligand C and so should appear at higher field.

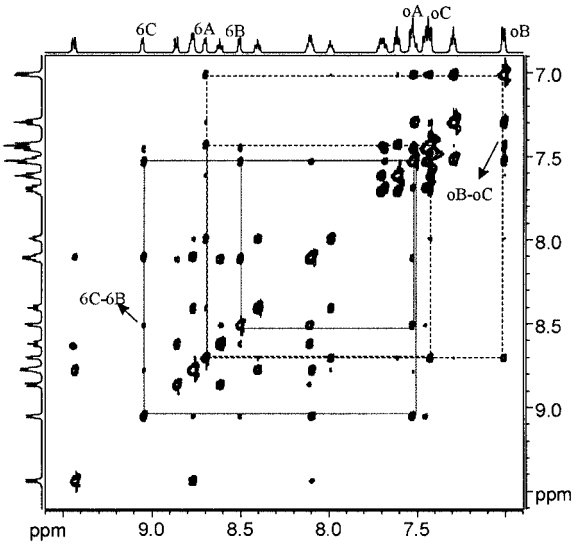

Figure 6. ^1H-^1H NOESY spectrum (600 MHz) and some assignments of the aromatic region of **1** in $[D_6]$acetone, 183 K. The dashed lines indicate the 6A-oB and 6A-oC NOEs. The dotted lines indicate the oA-6C and oA-6B NOEs. Arrows indicate the NOEs 6C-6B and oB-oC.

Two other NOE cross peaks observed in the 2D NOESY spectrum are the 6B-*o*A and 6C-*o*A NOEs. The first one is due to the α configuration of the ligands A and B, the second one is due to the ε configuration of ligands C and A.

NMR Characterization of 2

Interestingly, the ^1H NMR spectrum of **2** at room temperature shows partly sharp and partly broadened resonances (Figure 5). Cooling the NMR sample to 195 K all resonances sharpen. The ^1H NMR spectrum of **2** at 195 K shows only two sets of pyridine rings and one set of phenyl ring signals. Due to the C_2 axis present in this complex, the two azpy ligands are equivalent (A = B) and even the two pyridine rings of the bpy ligand are indistinguishable (see Figure 4). It appears to be difficult to assign the two sets of pyridine rings to either the bpy or the azpy ligand. An indication to assign properly the pyridine rings to either the azpy or the bpy ligand, is that the resonances of one pyridine ring are broadened at room temperature, whereas the other set of pyridine resonances is sharp at room temperature (Figure 5). As less broadening occurs introducing a

bpy ligand in these $[RuL_2L']^{2+}$ complexes (vide infra) it seems reasonable to assume that the broadened resonances correspond to the azpy ligand. Therefore, from the VT NMR studies (Figure 5) it follows that the sharp (at all temperatures) resonance at around 9.1 ppm corresponds to the H3 of bpy. Using COSY connectivities the other resonances are assigned.

The two most important NOE cross peaks that appear in the spectrum of **2** are (Figure S2, supp. inf.): 1) A NOE between the H6 resonance of one azpy ligand and the *ortho* resonance of the other azpy ligand (H6A–*o*B=H6B–*o*A) as A and B are forming the α configuration, and 2) a NOE between the H6 resonance of the bpy ligand and the *ortho* resonance of azpy (H6C-*o*A) which is a NOE due to the ε configuration (vide supra).

NMR Characterization of 3

The ^{1}H NMR assignment of β-$[Ru(azpy)_2(bpy)](PF_6)_2$ (**3**) is based on almost the same reasonings as used for **1**. The ^{1}H NMR of **3** (600 MHz) at room temperature shows all sharp resonances. However, also for this compound some signal broadening was observed at certain concentrations and temperatures, albeit not as extensively broadened as for example compound **1** (data not shown). Compound **3** consists again of pairs of ligands derived from known bis(ligand) moieties (Figure 3), which again can perfecly be used to assign the resonances properly. Ligand A and B form the α-Ru(azpy)(bpy) moiety. The 1D and 2D NOE data of α-$[Ru(azpy)(bpy)Cl_2]$ are just recently published.[29] Ligand B and C form the β-$[Ru(azpy)_2]$ moiety. Pyridine ring A (without prime) and ligand C form part of the ε-$Ru(azpy)_2$ backbone. In the 2D NOESY NMR spectrum (Figure S3, supp. inf.) one of the H6 resonances gives NOE cross peaks to two *ortho* resonances. These NOEs are assigned as the 6A-*o*B and 6A-*o*C NOEs in correspondence with the known cross peaks (H6-*o*) of the α-Ru(azpy)(bpy) moiety and ε-$Ru(azpy)_2$ configuration. Again the *o*B resonance is at higher field than the *o*C resonance as the *o*B hydrogens are in the shielding cone of pyridine ring A. Using the bpy H3-H3′ NOE, the other bpy pyridine ring can be assigned. The resonance 6A′ couples to two six resonances, i.e. 6B and 6C. As the chemical shifts of the protons 6B and 6C are almost identical they could not be assigned based on (de)shielding effects. Consequently, pyridine rings B and C could not be distinguished.

NMR Characterization of 4

The ^{1}H NMR of **4** shows all sharp resonances at room temperature and they remain sharp in the whole used temperature range. The ^{1}H-^{1}H 2D NOESY spectrum is shown in Figure S4, supp. inf. The H3-H3′ NOE of the bpy ligands has been used to assign the two bpy ligands. A simple ball and stick model shows that one of the bpy ligands has both its H6 hydrogen atoms in the shielding region of a pyridine ring, so the corresponding H6 resonances should both appear at high field. The other bpy ligand should have one H6 resonance at low field and one at high field (one H6 atom is in the shielding cone of a pyridine ring whereas the other H6 atom is above the azo bond, which gives less shielding). This allows the assignment of the resonances at δ = 8.05 and 7.72 ppm as H6A and H6A′ and the resonances at δ = 8.39 and 8.02 ppm as 6C and 6C′. Both H6C and H6A resonances give NOE cross peaks to the *ortho* resonance of the azpy ligand in agreement with the proposed structure of **4**.

Apparently, from the above-mentioned NMR spectroscopic data it is clear that the assignment of these kind of tris(ligand) complexes is quite complicated and the ^{1}H NMR assignment in literature of these kind of complexes is confusing, incomplete and sometimes erroneous.[19,21]

Variable Temperature and Concentration Studies

Although as yet not studied in detail, the concentration and temperature-dependent NMR spectra (300 MHz) show interesting differences between the complexes **1**, **2**, **3** and **4**. The temperature series of **1** at the lower concentration (Figure 5) results in sharper resonances at 193 K than in case of the higher concentration. The series of ^{1}H NMR spectra at various temperatures of **2** show less broadening in comparison to **1** and the differences between the two concentrations of **2** seem smaller. Finally the series of spectra of **3** also do show some signal broadening, which is comparable to **2**; one set of sharp resonances and one set of broadened reasonances (data not shown). In general, the comparison of **1** with **2** and **3** lead to the suggestion that introducing a bpy ligand in such tris(ligand) complexes results in a more rigid structure and consecutively less broadening. This is in agreement with the fact that the complexes $[Ru(azpy)(bpy)_2](PF_6)_2$ and $[Ru(bpy)_3](PF_6)_2$ do not show any signal broadening. Signal broadening in NMR occurring for this type of tris(ligand)ruthenium(II) complexes is not known in literature. For example in case of the related complex $[Ru(abpy)_3]^{2+}$ [(abpy = 2,2-azobis(pyridine)] ^{1}H NMR spectra data are reported, but no broadening has been mentioned.[28]

One mechanism that may be responsible for signal broadening in (azpy)ruthenium complexes is sterically hindered rotations of one or more phenyl rings.[30] In theory this implies that upon cooling the sample during the NMR experiment, the phenyl ring rotation becomes slower on the NMR time scale and at the lowest temperatures it is possible to stop the phenyl ring rotation (on the NMR time scale). This would result in distinct resonances for the *o* and *m* protons on one side of the phenyl ring and the *o* and *m* protons on the other side. However, the fact that such distinct resonances were not observed for any of the azpy-containing (tris)ligand complexes indicates that hindered rotations of phenyl rings are not responsible for the observed signal broadening. However, hindered tolyl-ring rotations might still be present in *mer*-$[Ru(tazpy)_3](PF_6)_2$, since upon cooling and diluting the NMR sample of this compound, no conditions were found which resulted in sharp resonances.

The fact that the broadening of the resonances of the tris(ligand) complexes is also dependent on concentration might suggest stacking or intermolecular interactions.

The possibility of ligand exchange/decoordination can be ruled out by the fact that no free ligand was observed at low temperatures and the fact that addition of free ligand did not influence the broadening of the signals.

Another explanation for the VT NMR results appears to lie in intramolecular rearrangements, which are known in the literature[31] to be able to result in NMR signal broadening. Nevertheless more research is needed to comprehend this phenomenon of signal broadening.

Cytotoxicity Tests

The complexes **1**, **2**, **3** and **4** have been tested for their cytotoxicity against a series of human tumor cell lines. The data are summarized in Table 6. Compound **1** shows in the cell lines EVSA-T and M19 quite a significant cytotoxicity, but in the other cell lines the IC_{50} values are moderate to low. Compound **2** exhibits in EVSA-T, M19 and MCF-7 a considerable cytotoxicity. Compound **3** displays only in the cell line M19 a considerable cytotoxicity and compound **4** still shows a considerable cytotoxicity in the cell lines EVSA-T and H226. Interesting to mention is that the compound $[Ru(bpy)_3](PF_6)_2$ does not show any cytotoxic effect under these circumstances (data not shown). It is important to note that the activity of these complexes cannot be caused by traces of starting materials, like for example α-$[Ru(tazpy)_2(NO_3)_2]$ or azpy. The former one shows an activity of approximately 8 μM[32] and the ligand azpy shows no cytotoxicity at all.[33] It is quite remarkable that this series of compounds **1**, **2**, **3** and **4** show a moderate cytotoxicity in some cell lines,[33] as these complexes do not have any anionic (chloride) ligands, to allow, after hydrolysis, coordination to DNA bases. This behavior might point to a class of very interesting ruthenium complexes acting by another mechanism than cisplatin and therefore opening the way to a new category of anticancer agents, agents which might be successful against cancer types where the old generation drugs appeared to be inactive. The activity of these tris(chelated) complexes might be caused by an intercalative interaction with DNA rather than a cisplatin-like covalent interaction with DNA. Interestingly just recently a publication appeared[34] concerning a series of tris(ligand) complexes $[Ru(L)_2(Y)]Cl_2$ with L bpy or phen and Y thiosemicarbazone-type of ligands. These complexes show a considerable antitumor activity in mice and therefore strengthening the fact that tris(ligand) complexes are a very important group of potential new ruthenium anticancer drugs.

Conclusions

Tris(ligand)ruthenium(II) azpy-like complexes show in many aspects interesting properties. The NMR spectroscopic data of the series of complexes $[RuL_3](PF_6)_2$ (L = 2-phenylazopyridine or *o*-tolylazopyridine) and $[RuL'_2L''](PF_6)_2$ (L′ and L′′ are 2-phenylazopyridine or 2,2′-bipyridine) show broadening of the resonances for especially the complexes **1**, **2** and **5**. This broadening appears to depend on temperature, concentration and steric hindrance within the complex. Choosing the right conditions, sharp resonances have been obtained and the complicated NMR spectra of these tris(ligand) complexes could be almost completely resolved. Especially the fact that these tris(chelated) complexes are build of pairs of known configurations (e.g. α-, β-, and ε-$Ru(azpy)_2$) with previously determined (2D) NMR spectroscopic data, allowed the assignment of almost all resonances of the tris(ligand) complexes.

The cytotoxicity data show that the complexes *mer*-$[Ru(azpy)_3](PF_6)_2$, α-$[Ru(azpy)_2(bpy)](PF_6)_2$, β-$[Ru(azpy)_2(bpy)](PF_6)_2$, and $[Ru(azpy)(bpy)_2](PF_6)_2$ have a moderate cytotoxicity in some particular cell lines. In general, the fact that these fully coordinated tris(chelated) complexes still show some activity, suggests intercalative properties responsible for eventual cell death. In conclusion, these complexes appear to act through another mechanism than the compound cisplatin, which indicates that in general these ruthenium 2-phenylazopyridine complexes are a very interesting class of anticancer complexes, conceivably being able to lead to a different type of anticancer drugs.

Experimental Section

Syntheses: The syntheses of the tris(ligand)ruthenium(II) complexes mentioned below are modifications of literature syntheses[12,13,22] for *mer*-$[Ru(azpy)_3]^{2+}$, $[Ru(azpy)_2(bpy)]^{2+}$ and $[Ru(azpy)(bpy)_2]^{2+}$ using *cis*-$[Ru(bpy)_2Cl_2]\cdot 2H_2O$ or α- and β-$[Ru(azpy)_2(NO_3)_2]$ as starting materials.[23,24,35]

***mer*-$[Ru(azpy)_3](PF_6)_2$ (1):** α-$[Ru(azpy)_2(NO_3)_2]$ (40.0 mg, $6.76\cdot10^{-5}$ mol) and azpy (37.4 mg, $2.04\cdot10^{-4}$ mol) were dissolved in water (5 mL) and methanol (5 mL). The red solution was refluxed for

Table 6. IC_{50} values [μM] of the tris(chelated) ruthenium complexes **1**, **2**, **3** and **4**.

Tested compound	A498	EVSTA-T	H226	IGROV	M19	MCF-7	WIDR
1	55.4	12.8	45.6	32.1	4.47	25.2	29.2
2	89.8	14.6	46.5	62.6	13.3	17.9	23.2
3	92.0	38.7	65.2	54.6	19.5	52.4	64.4
4	77.8	20.7	17.7	63.8	25.9	40.4	87.0
α-$[Ru(azpy)_2Cl_2]$	0.27	0.063	0.48	0.27	0.064	0.27	0.27
Cisplatin	7.5	1.4	10.9	0.6	1.9	2.3	3.2
5-Fluorouracil	1.1	3.7	2.6	2.3	3.4	5.8	1.7

one hour, during which time the solution turned red-brown. After cooling to room temperature, a concentrated aqueous solution of NH_4PF_6 (240 mg) was added, which led to the precipitation of a brown product. The brown product was washed with water and dried with diethyl ether, resulting in a yield of 38.0 mg (75%). $C_{33}H_{27}F_{12}N_9P_2Ru$ (940.1): calcd. C 42.1, H 2.9, N 13.4; found C 42.1, H 3.0, N 13.2.

α-[Ru(azpy)$_2$(bpy)](PF$_6$)$_2$ (2): α-[Ru(azpy)$_2$(NO$_3$)$_2$] (30.0 mg, $5.07\cdot10^{-5}$ mol) and bpy (23.8 mg, $1.47\cdot10^{-4}$ mol) were dissolved in water (5 mL) and methanol (5 mL). The purple solution was refluxed for 90 minutes, resulting in a brown solution. After cooling to room temperature, a concentrated aqueous solution NH_4PF_6 was added, which led to the precipitation of a red-brown product. This product was washed with water and diethyl ether and dried in vacuo with P_4O_{10}. Yield: 45.7 mg (99%). $C_{32}H_{26}F_{12}N_8P_2Ru$ (913.1): calcd. C 42.1, H 2.9, N 12.3; found C 43.6 H 3.0, N 12.6.

β-[Ru(azpy)$_2$(bpy)](PF$_6$)$_2$ (3): β-[Ru(azpy)$_2$(NO$_3$)$_2$] (20.0 mg, $3.38\cdot10^{-5}$ mol) and bpy (15.8 mg, $1.0\cdot10^{-4}$ mol) were refluxed for 1.5 hours in a mixture of water (3.5 mL) and of methanol (3.5 mL). During this time a color change from purple to red occurred. The resulting solution was filtered and a concentrated aqueous solution of NH_4PF_6 was added. A brown crystalline material formed within an hour. This product was collected by filtration and washed with water and diethyl ether. Yield: 22.7 mg (74%). $C_{32}H_{26}F_{12}N_8P_2Ru$ (913.1): calcd. C 42.1, H 2.9, N 12.3; found C 41.7, H 3.0, N 13.2.

[Ru(azpy)(bpy)$_2$](PF$_6$)$_2$ (4): *cis*-[Ru(bpy)$_2$Cl$_2$]·2H$_2$O (25.0 mg, $4.80\cdot10^{-5}$ mol) and azpy (44.0 mg, $2.40\cdot10^{-4}$ mol) were refluxed for 3.5 hours in water (5 mL), during which the solution changed color from yellow to red. The reaction mixture was filtered and a concentrated aqueous solution of NH_4PF_6 was added. A red precipitate could be collected, which was subsequently washed with small amounts of water and diethyl ether. Yield: 38.3 mg (90%). $C_{32}H_{25}F_{12}N_7P_2Ru\cdot1.5H_2O$ (925.1): calcd. C 40.8, H 3.1, N 10.8; found C 40.5, H 2.7, 10.9.

***mer*-[Ru(tazpy)$_3$](PF$_6$)$_2$ (5):** The precursor material α-[Ru(tazpy)$_2$(NO$_3$)$_2$] was prepared analogous to the synthesis[24] of α-[Ru(azpy)$_2$(NO$_3$)$_2$] with use of the ligand *o*-tolylazopyridine. α-[Ru(tazpy)$_2$(NO$_3$)$_2$] (20.0 g, $3.23\cdot10^{-5}$ mol) was refluxed with tazpy (19 mg, $9.6\cdot10^{-5}$ mol) in a mixture of methanol (5 mL) and water (5 mL) for one hour. A color change from violet to brown was noted and the solution was cooled to room temperature. A concentrated solution of NH_4PF_6 was then added, resulting in a brown precipitate, which was collected, washed with water, and dried in vacuo with P_4O_{10}. Yield: 24.6 mg (78%). $C_{36}H_{33}F_{12}N_9P_2Ru$ (982.1): calcd. C 44.00, H 3.38, N 12.83; found: C 44.0, H 3.1, N 12.9.

X-ray Crystal Structure Determination of 1 and 5: Crystals of suitable size were placed in the cold nitrogen stream of a Nonius KappaCCD diffractometer on rotating anode. Data were collected at 150 K, using Mo-K_α radiation (graphite monochromator, λ = 0.71073 Å). For **5** an absorption correction based on multiple measurements of symmetry-related reflections was applied (PLATON/MULABS,[36] T-range 0.898–0.950). The structures were solved by direct methods (SHELXS86,[37]) and refined on F^2 using full-matrix least-squares techniques (SHELXL-97-2,[38]). One of the PF_6 counterions in the crystal structure of **1** was refined with a two-site disorder model. Mild distance restraints were applied to enforce a reasonable geometry of the minor disorder component. Hydrogen atoms were included in the model on calculated positions, riding on their carrier atoms. The methyl groups of **5** were rotated along the C–C bond during refinement. All non-hydrogen atoms were refined with anisotropic displacement parameters with exception of the minor component atoms of the disordered counterion in **1**. Hydrogen atom displacement was described with a fixed isotropic parameter linked to the equivalent isotropic parameter of the carrier atom. Pertinent data for each of the structure determinations are given in Table 1. CCDC-251700 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Cyrstallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Physical Measurements: The NMR measurements were performed with a Bruker 300 DPX spectrometer and a 600 DPX spectrometer. Spectra were recorded in [D$_6$]acetone and calibrated on the solvent peak. 2D ^{1}H-^{1}H NOESY spectra were performed using the standard Bruker pulse sequences, with a mixing time of 0.8–1 s.

Cytotoxicity Tests. Determination IC$_{50}$ Values: For the determination of the IC_{50} values of the compounds **1**, **2**, **3** and **4** a panel of human tumor cell lines (MCF-7, EVSA-T, WIDR, IGROV, M19, A498 and H266) has been used. The in vitro cytotoxicity assays using these cell lines were performed at the Dr. Daniel den Hoed kliniek (Rotterdam Cancer Institute), Department of Medical oncology (Rotterdam, The Netherlands).

A stock solution of the test and reference compounds was used, which contained 1 mg compound/200 μL. The compounds were dissolved in DMSO. On the first day 150 μL of trypsinized tumor cells (1500–2000 cells/well) were plated in 96-wells flatbottom microtiter plates. The plates were preincubated 48 h at 37 °C, 8.5% CO_2 to allow the cells to adhere. On day 2, a threefold dilution sequence of ten steps was made in full medium, starting with the 1 mg compound/200 μL stock solution. Every dilution was used in quadruplicate by adding 50 μL to a column of four wells. On day 7 the incubation was terminated by washing the plate twice with PBS. Cytotoxicity was estimated by the microculture sulforhodamine B (SRB) test.[39] The absorbance was read at 540 nm using an automatic microplate reader (labsystems Multiskan MS). Data were used for construction of concentration response curves and determination of the IC_{50} value by use of Deltasoft 3 software.

Supporting Information (see also footnote on the first page of this article): ^{1}H NMR spectrum of **1** (Figure S1), 2D NOESY spectra of **2–4** (Figure S2–S4) and Table S1 showing NOE cross peaks for **1–4** (see also footnote on the first page of this article).

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