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Synthesis, characterization and DNA binding properties of oligopyridine-ruthenium(II)-amino acid conjugates

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Abstract

The DNA-binding properties of a number of ruthenium oligopyridine complexes with conjugated amino acids having the general formulae $[Ru(terpy)(4\text{-}COY\text{-}4'\text{-}Mebpy)(X)]^{n+}$, X = NO ($n = 3$), X = Cl ($n = 1$) and NO_2 ($n = 1$) and Y = $AlaCONH_2$ and $TrpCONH_2$ are reported. The new complexes were spectroscopically characterized and their DNA-binding properties were studied by means of circular dichroism (CD), ^{23}Na and ^{31}P NMR spectroscopy. The results show that the chlorido complexes interact by coordination to the DNA bases with the conjugated amino acid able to provide an additional interaction with the DNA helix. In addition, electrostatic interactions between all studied complexes and the DNA polyanion were observed. The nitro complexes were found to be insignificant, affecting only the ^{31}P NMR signal, probably due to changes in the hydration sphere of the DNA close to the phosphates.

Keywords: Ruthenium complexes; DNA interaction; ^{23}Na and ^{31}P NMR spectroscopy

1. Introduction

The investigation on Ruthenium compounds able to bind coordinatively to DNA is one of the most attractive research fields for the development of novel non-platinum anticancer agents [1–3]. Chloro-amino [4], chloro-polypyridyl [5], nitrosylo-chloro-polypyridyl [6], and dimethylsulfoxide ruthenium complexes [3] are among the most studied compounds regarding their DNA-binding affinity and their cytostatic properties. Also, the photoreactivity of Ru(II) tris-chelates which bind electrostatically to DNA helix, has been the subject of research interests in recent years [7–9]. Such types of complexes have been proposed as therapeutic agents in cancer photodynamic therapy (PDT) [10]. The DNA damages caused by these complexes originate either from energy transfer, leading to the production of singlet oxygen ($^1O_2$) [11] or from a direct photoelectron transfer process from a DNA base to the excited state of the complex [11]. Among the most studied compounds is the complex $[Ru(bpy)_3]^{2+}$, which shows a weak association with DNA, most probably due to the electrostatic interactions with the DNA phosphates [12–14]. Attempts to undertake the random binding of ruthenium tris-chelates to DNA have been raised by tethering a DNA-recognition peptide to one of the bipyridine ligands [15–23]. It has been suggested that the nature of the peptide affects the DNA binding affinity of the ruthenium complex [15,17,18].

Since the DNA photocleavage process mainly depends on the distance between the bound complex and the DNA helix, it has been proposed that Ru(II) polypyridine complexes, which bind firmly (e.g. intercalators) to the polynucleotide chain, are the most efficient compounds to cleave the DNA strands [24]. On the other hand the complex

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$[Ru(NH_3)_4(NO_2)P(OEt)_3]^+$, a non-classical DNA binder, upon light irradiation releases the $(NO_2)^-$ anion, producing NO· and free radicals of $O^{\cdot -}$ or HO·, known to be able to cause DNA cleavage [25,26]. Moreover, photoinduced DNA cleavage by the complex [Ru(terpy)(4-CO_2H-4′-Mebpy)(NO_2)]$^+$ has been observed [24]. Both complexes are not tightly bound to DNA and for this reason a mechanism, generated from the photorelease of the $(NO_2)^-$ anions probably through free radicals, has been suggested [25,26].

In an attempt to investigate the DNA binding affinity of the ruthenium oligopyridine complexes with conjugated amino acids, with respect to the amino acid nature, a series of new complexes of general formulae [Ru(terpy)(4-CO_2Y-4′-Mebpy)(X)]$^{n+}$, X = NO ($n = 3$), X = Cl ($n = 1$) and NO_2 ($n = 1$) and Y = AlaCONH$_2$ and TrpCONH$_2$ have been synthesized, characterized and their DNA binding properties have been studied. CD, ^{23}Na and ^{31}P NMR spectroscopy was used to determine the DNA changes, caused by the complexes' interactions. The ^{23}Na nuclear spin relaxation is an important technique to investigate the interactions of positive charged ions or drugs with DNA [27–30]. Also, ^{31}P chemical shift of the DNA phosphate bond is a valuable addition technique for monitoring conformational changes in DNA [31]. The ^{31}P chemical shift is sensitive to interactions of metal complexes with DNA [32,33]. The two amino acids chosen contain an aromatic and an aliphatic side chain and their comparison was attempted.

2. Experimental

2,2′:6′,2″-Terpyridine (terpy) was purchased from Aldrich Chemical Company and used without further purification. The complex Ru(terpy)Cl_3 [34] and the ligand 4-carboxy-4′-methyl-2,2′-bipyridine (4-CO_2H-4′-Mebpy) [23] were prepared according to literature procedures. Complex [Ru(terpy)(4-CO_2H-4′-Mebpy)Cl](PF$_6$) was prepared and purified by a literature method [35]. The resin (TentaGel S RAM) for the amino acid immobilization and the protected amino acids Fmoc-Trp-(Boc)-OH, Fmoc-His(Trt)-OH and Fmoc-Ala-OH were purchased from Rapp Polymere Ltd. and CBL Patras Ltd., respectively. Sonicated calf thymus DNA was prepared according to the literature [36]. The length of the fragments after the sonication, which were used for the NMR and CD experiments, was checked by size exclusion chromatography and a dispersion of DNA lengths from 70 bp to 300 bp was found. The DNA concentration, expressed as moles of nucleotides per litre [P], was determined on a Pharmacia LKB-Biochrom 4060 UV–visible spectrophotometer from the absorbance at 260 nm ($\varepsilon_{260} = 6600\ M^{-1}\ cm^{-1}$, $T =$ 298 K). The sample was found to be consistent with low protein content as the value of the A_{260}/A_{280} ratio was 1.9. UV–Visible spectra of the complexes and DNA titration were recorded on a Varian Cary 3, spectrophotometer. Thermal denaturation experiments were performed in quartz cuvettes. Samples were continuously heated with a 1 °C min^{-1} rate of temperature increase, while monitoring the absorbance changes at 260 nm. The investigated interval of temperature ranged from 24 to 98 °C. Values for melting temperatures (T_m) were determined according to the reported procedures [24]. The infrared spectra of the complexes were recorded on a Perkin–Elmer Paragon1000 FTIR spectrophotometer, equipped with a Golden Gate Diamond ATR device, using the diffuse reflectance technique. C, H and N determinations were performed on a Perkin–Elmer 2400 Series II analyzer. The ^{1}H, ^{23}Na and ^{31}P NMR spectra were obtained on a Bruker 300 DPX spectrometer operating at 300.13 MHz, 121.49 MHz and at 79.39 MHz, respectively, using TMS (for ^{1}H) and H_3PO_4 (for ^{31}P) as external standards. ^{23}Na and ^{31}P NMR spectral changes were observed upon addition of complexes (**1**), (**2**), (**5**) and (**6**) to calf thymus DNA (70–300 bp), at ratios 0.1–0.5 (DNA concentration = 6 mM, $T = 298$ K, pH 7.01).

CD spectra were recorded on a Jobin Yvon CD-6 and on JASCO V-715 dichrograph equipped with a temperature controller at 25 °C. Circular dichroism spectral changes were observed upon addition of the complexes to calf thymus DNA (70–300 bp) at ratios 0.1, 0.3, 0.5 (DNA concentration 100 μM, $T = 25$ °C, pH 7.01).

2.1. Synthesis of 4-COY-4′-Mebpy (Y = AlaCONH$_2$, TrpCONH$_2$)

The immobilization of the Fmoc-protected amino acids (Fmoc-Trp-(Boc)-OH, and Fmoc-Ala-OH) on the resin was performed with a standard Fmoc protocol [37]. The conjugation of the ligand 4-CO_2H-4′-Mebpy (0.5 mmol) to the resin-bound aminoacid was achieved with the coupling agents benzotriazol-1-yl-oxytris(pyrrolidino)phosphanium hexafluorophosphate (PyBOP, 0.75 mmol) and diisopropylethylamine (DIPEA, 1 mmol).

In a typical experiment 500 mg of resin bound amino acid (substitution 0.22 mmol/g) was treated with 20% piperidine solution in NMP (5 mL), to deprotect the Fmoc group of the amino acid. In the resulted resin bound deprotected amino acid, a solution of 4-CO_2H-4′-Mebpy (0.5 mmol) in NMP (2 mL) containing DIPEA (1 mmol) and PyBOP (0.5 mmol) was added and remained to react for 3 h at room temperature. Then, the resin was filtered, washed with NMP (5×5 mL) and CH_2Cl_2 (2×5 mL). The purity of the ligand 4-COY-4′-Mebpy, after the cleavage from the resin with TFA/H_2O (95/5, v/v), was checked with ^{1}H NMR and LC–MS spectrometry. 4-COAlaCONH$_2$-4′-Mebpy, Yield: (~80%) *Anal.* Calcd. for $C_{15}H_{16}N_4O_2$: C, 63.4; H, 5.7; N, 19.7. Found: C, 63.2; H, 5.7; N, 19.4%.

4-COTrpCONH$_2$-4′-Mebpy, Yield: (~68%) *Anal.* Calcd. for $C_{23}H_{21}N_5O_2$: C, 69.2; H, 5.3; N, 17.5. Found: C, 68.9; H, 5.7; N, 17.4%.

2.2. Synthesis of the complexes [Ru(terpy)(4-COY-4′-Mebpy)Cl](PF$_6$), (Y = AlaCONH$_2$, TrpCONH$_2$) (**1**), (**2**)

The two complexes were prepared in a similar way. An amount (0.11 mmol) of the resin bound ligand, 4-COY-4′-Mebpy, was refluxed with an excess of Ru(terpy)Cl$_3$ (0.33 mmol), LiCl (0.36 mmol, ~15 mg) and Et$_3$N (0.25 mL), in DMF/EtOH (3:1) for 7 h. The resin with the immobilized complexes was carefully washed with DMF (5 × 5 mL) to remove the unreacted Ru(terpy)Cl$_3$ and then dried with CH_2Cl_2 (3 × 5 mL).

The cleavage of the complex and the protecting groups from the resin was achieved using 5 mL of TFA/H_2O (95/5, v/v). 40 mL of diethyl ether was added to the resulted solution, and was kept at 7 °C overnight to afford a red-brown precipitate which was filtered, dissolved in 1 mL of methanol and added to a saturated aqueous solution of NH_4PF_6. The methanol was removed by evaporation and the complex was precipitated as PF_6^- salt, washed with cold acetone (2 × 5 mL) and dried in vacuo.

(1) [Ru(terpy)(4-COAlaCONH$_2$-4′-Mebpy)Cl](PF$_6$). Yield: (~75%). *Anal.* Calcd. for $C_{30}H_{27}ClF_6N_7O_2$-PRu: C, 45.1; H, 3.4; N, 12.3. Found: C, 45.3; H, 3.4; N, 12.1%. ESI-MS: *m/z,* 654.

(2) [Ru(terpy)(4-COtrpCONH$_2$-4′-Mebpy)Cl](PF$_6$). Yield: (~60%). *Anal.* Calcd. for $C_{38}H_{32}ClF_6N_8O_2$: C, 49.9; H, 3.5; N, 12.3. Found: C, 49.6; H, 3.4; N, 12.1%. ESI-MS: *m/z,* 769.

2.3. Synthesis of the complexes [Ru(terpy)(4-COY-4′-Mebpy-Y)(NO)](PF$_6$)$_3$, (Y = AlaCONH$_2$, TrpCONH$_2$), (**3**), (**4**)

The two complexes were prepared similarly. An amount of 0.68 mmol of [Ru(terpy)(4-COY-4′-Mebpy)Cl](PF$_6$)$_3$ was suspended in 10 ml of water and the solution was refluxed for 15 min. The deep red-brown solution was filtered hot and then cooled to room temperature. The pH of the solution was adjusted around ~2 with the appropriate amount of hydrochloric acid 0.1 M HCl. One milliliter of aqueous solution containing 47 mg (0.68 mmol) of sodium nitrite $NaNO_2$ was added slowly over a 10 min period. After complete addition, the brown red solution became yellow and was warmed at 60 °C for an additional time of 45 min. A methanolic solution (4 mL) of saturated NH_4PF_6 was added and the precipitate was collected by filtration, washed with diethyl ether (3 × 5 mL) and dried in vacuo.

(3) [Ru(terpy)(4-COAlaCONH$_2$-4′-Mebpy-Y)(NO)](PF$_6$)$_3$. Yield: (~60%). *Anal.* Calcd. for $C_{30}H_{27}F_{18}N_8O_3$-P_3Ru: C, 33.2; H, 2.5; N, 10.3. Found: C, 32.9; H, 2.4; N, 10.1%. ESI-MS: *m/z,* 649.

(4) [Ru(terpy)(4-COTrpCONH$_2$-4′-Mebpy-Y)(NO)](PF$_6$)$_3$. Yield: (~60%). *Anal.* Calcd. for $C_{38}H_{32}F_{18}N_9O_3$-P_3Ru: C, 38.1; H, 2.7; N, 10.5. Found: C, 37.9; H, 2.6; N, 10.3%. ESI-MS: *m/z,* 764.

2.4. Synthesis of the complexes [Ru(terpy)(4-COY-4′-Mebpy)(NO$_2$)](PF$_6$)$_2$, (Y = AlaCONH$_2$, TrpCONH$_2$,) (**5**), (**6**)

These two complexes were prepared similarly. An amount of 0.68 mmol of [Ru(terpy)(4-COY-4′-Mebpy)Cl](PF$_6$) was suspended in 10 mL of water and the solution was refluxed for 15 min. The deep red-brown solution was filtered hot and then cooled to room temperature. The pH of the solution was adjusted around ~10 with 0.1 M NaOH and then 1 mL of an aqueous solution containing 47 mg (0.68 mmol) of sodium nitrite $NaNO_2$ was added dropwise over a 10 min period. The brown-red solution became orange and was warmed at 60 °C for an additional time of 30 min. After cooling at room temperature, a methanolic solution (4 mL) of saturated NH_4PF_6 was added. The precipitate was collected by filtration, washed with diethyl ether (3 × 5 mL) and dried in vacuo.

(5) [Ru(terpy)(4-COAlaCONH$_2$-4′-Mebpy)(NO$_2$)](PF$_6$)$_2$. Yield: (~50%). *Anal.* Calcd. for $C_{30}H_{27}F_6N_8O_4$PRu: C, 44.5; H, 3.4; N, 13.8. Found: C, 44.7; H, 3.4; N, 14.1%. ESI-MS: *m/z,* 649.

(6) [Ru(terpy)(4-COTrpCONH$_2$-4′-Mebpy)(NO$_2$)](PF$_6$)$_2$. Yield: (~50%). *Anal.* Calcd. for $C_{38}H_{32}F_6N_9O_4$PRu: C, 49.4; H, 3.5; N, 13.6. Found: C, 49.1; H, 3.5; N, 13.3%. ESI-MS: *m/z,* 780.

3. Results and discussion

3.1. Synthesis and characterization of complexes (**1**)–(**6**)

Chlorido-complexes (**1**) and (**2**) were prepared through the solid-phase synthesis strategy, according to a previously published procedure [16]. All the prepared complexes were isolated as two positional isomers, differing in the orientation of the ligand 4-COY-4′-Mebpy (Y = AlaCONH$_2$, TrpCONH$_2$), towards coordination to the ruthenium center. The separation of the isomers was only partially achieved chromatographically (Sephadex LH-20) in an approximate ratio 1:3. The predominant major isomer in each case was characterized; however the solutions of 1:1 ratio were used for all the other studies. Complexes (**3**) and (**4**) were synthesized by the replacement of the chloride in the corresponding chlorido-complexes by NO at acidic pH (pH ~ 2). The nitro complexes (**5**) and (**6**) were obtained in basic conditions (pH ~ 10).

The IR spectra of complexes (**3**) and (**4**) show a strong and sharp IR band at 1949 and 1900 cm^{-1}, respectively, which was assigned to the stretching vibration ν(NO). The ν(NO) stretching frequencies were similar to those

observed in the complex $[Ru(terpy)(bpy)NO]^{3+}$ (1952 cm^{-1}) [38]. However, the nature of the conjugated amino acid seems to affect the value of the ν(NO) stretching frequency, which is shifted to lower frequencies in the order $AlaCONH_2 < TrpCONH_2$. It has been suggested that the ν(NO) value is a good indicator of the degree of positive charge residue on the coordinated ruthenium NO. This is also associated with the electrophilic reactivity of the nitrosyl group [39]. Thus, it seems that the amino acid conjugation to the ligand 4-COY-4′-Mebpy increases the electrophilicity of the NO from complex (**3**) to (**4**). In the case of complex [Ru(terpy)(4-CO-gly-L-ala-L-lys-$CONH_2$-4′-Mebpy)NO](PF6)$_3$ the ν(NO) was observed at 1924 cm^{-1}, indicating that the electrophilicity decreases in comparison with the complex $[Ru(terpy)(bpy)\ NO]^{3+}$ [24] (1952 cm^{-1}). In the IR spectra of the nitro complexes (**5**) and (**6**) the absence of the ν(NO) stretching vibration band, indicates the complete transformation of the corresponding nitrosyl complexes to the nitro ones.

In the electronic spectra of complexes (**1**) and (**2**) in aqueous solutions, the intense bands at 249 and 269 nm correspondingly, are most likely intraligand (bpy and terpy) $\pi \rightarrow \pi^*$ transitions [38,40]. The bands at 299 and 310 nm in the spectrum of complex (**1**) and (**2**) correspondingly are probably MLCT transitions $d\pi Ru \rightarrow \pi^*$(ligand) [38,41,42]. In the electronic spectra of the corresponding nitrosyl complexes (**3**) and (**4**) in dry MeCN the lowest energy bands at 355 and 360 nm correspondingly, are attributed to the $d\pi Ru \rightarrow \pi^*(NO)$ transition [6,24]. It has been suggested that the $d\pi Ru \rightarrow \pi^*(NO)$ transition band reflects the destabilization of the dπ level, due to the $d\pi Ru \rightarrow \pi^*(NO)$ back bonding. Thus it affects the electrophilic reactivity of the NO [6,24]. Shifting at lower energies indicates a decrease of the electrophilicity of the NO. Comparing the observed band values with those of the complexes [Ru(terpy)(4-CO-gly-L-ala-L-lys-$CONH_2$-4′-Mebpy)NO](PF$_6$)$_3$ (350 nm) [6] and [Ru(terpy)(4-CO_2-4′-Mebpy)NO] (PF$_6$)$_3$ (339 nm) [6] we can assume that the influence of the nature of the conjugated ligand Y to the ligand 4-COY-4′-Mebpy follows the order H > gly-L-ala-L-lys-$CONH_2$ > L-ala > L-trp. This effect can be explained on the basis of the destabilization caused by the conjugate (Y) to the ligand 4-COY-4′-Mebpy ring system. The corresponding nitro complexes (**5**) and (**6**) display the MLCT band $d\pi Ru \rightarrow \pi^*(NO_2)$, red shifted at 474 and 478 nm correspondingly. The characteristic spectroscopic data are summarized in Table 1.

The ^{1}H NMR spectra of the chlorido complexes (**1**) and (**2**) in D_2O (buffer phosphates, pH 7.0) show distinct double resonances for the ligand's 4-CO_2Y-4′-Mebpy protons and slightly broad signals for the terpy, indicating the presence of two chemically non-equivalent species. The formation of two positional isomers, differing in the orientation of the ligand 4-CO_2Y-4′-Mebpy towards coordination to ruthenium centre, provides a possible explanation [35,16]. In their ^{1}H NMR spectra the resonances of the major isomer predominate in each case. The ^{1}H NMR spectrum of the complex (**3**) shows the same value of the resonances as those of complex (**5**); also those of complex (**4**) are the same as those of complex (**6**), indicating the conversion of the nitrosyl complexes to the corresponding nitro complexes under neutral pH conditions. TOCSY experiments were performed to assign the proton resonances. The lowest field doublet of the 4-CO_2Y-4′-Mebpy, is assigned to 4-CO_2Y-4′-Mebpy H6, since no correlation peak was observed with the 4′-Mebpy-protons. However, in the case of isomer II, the lowest field doublet correlates with the methyl protons signal, indicating that this proton belongs to the 4′-Mebpy pyridine ring system [16]. The full assignments of the proton signals are presented in Table 2. The structures of the positional isomers with the used atomic numbering are shown in Fig. 1.

Table 1
Characteristic spectral data for the complexes (**1**)–(**6**)

Complexes	IR (cm^{-1})			UV–Vis (nm)[a]
	ν(NO)	$\nu_{as}(NO_2)$	$\nu_s(NO_2)$	
(**1**)				249 $\pi \rightarrow \pi^*$ intraligand 299 $d_\pi(Ru) \rightarrow \pi^*$(terpy)
(**2**)				269 $\pi \rightarrow \pi^*$ intraligand 299 $d_\pi(Ru) \rightarrow \pi^*$(bpy) 310 $d_\pi(Ru) \rightarrow \pi^*$(terpy)
(**3**)	1949			270 $d_\pi(Ru) \rightarrow \pi^*$(terpy) 305 $d_\pi(Ru) \rightarrow \pi^*$(bpy) 355 $d_\pi(Ru) \rightarrow \pi^*$ NO
(**4**)	1900			268 $d_\pi(Ru) \rightarrow \pi^*$(terpy) 355 $d_\pi(Ru) \rightarrow \pi^*$ NO
(**5**)		1449	1294	270 $\pi \rightarrow \pi^*$ intraligand 279 $\pi \rightarrow \pi^*$ intraligand 310 $d_\pi(Ru) \rightarrow \pi^*$(terpy) 330 $d_\pi(Ru) \rightarrow \pi^*$(bpy) 474 $d_\pi(Ru) \rightarrow \pi^*$ NO_2
(**6**)		1445	1289	268 $\pi \rightarrow \pi^*$ intraligand 282 $\pi \rightarrow \pi^*$ intraligand 490 $d_\pi(Ru) \rightarrow \pi^*$ NO_2

[a] In H_2O for complexes (**1**) and (**2**) and dry CH_3CN for complexes (**3**)–(**6**).

3.2. DNA binding studies

In order to study the DNA interactions of the nitrosyl complexes in aqueous media, their stability at neutral pH was first investigated by spectroscopic methods. The electronic spectra of the nitrosyl complexes (**3**) and (**4**), are exactly the same as those of the nitro complexes (**5**) and (**6**), respectively. This result shows that the nitrosyl complexes (**3**) and (**4**) were transformed to the corresponding nitro ones under neutral pH.

The interactions of the chlorido-complexes (**1**) and (**2**) with CT-DNA were studied by circular dichroism, ^{23}Na and ^{31}P NMR spectroscopy. The CD spectra of calf thymus DNA, after the addition of complex (**1**) at ratio's $r = 0.1$, $r = 0.3$ and $r = 0.5$ ($r = [Ru]/[DNA]$), are shown

Table 2
^{1}H NMR (300 MHz) chemical shifts (δ with respect to TMS) for the isomeric complexes 298 K

Complexes	4-CO_2Y-4′-Mebpy							Terpy							α-Ala	β-Ala	α-trp	β-trp	δ-trp	ε,ζ,η-trp
	H6	H5	H3	H6′	H5′	H3′	4-Me	H3	H3′	H4	H4′	H5/H5′	H6/H6′	H3′′	H4′′	H5′′				
Mebpy-ala	8.83	7.84	8.42	8.55	7.81	8.38	2.61										4.60	3.30	7.41	7.15
Mebpytrpt	8.87	7.91	8.21	8.65	7.73	8.02	2.80													
(**1**) I	10.41	8.43	9.28	9.05	8.54	7.03	2.50	7.53	7.53	7.33	7.33	8.07	8.77	8.74	8.25	8.74	4.60	1.53		
II	9.03	8.60	7.41	10.07	7.99	8.98	2.12	7.78	7.78	7.41	7.41	8.01	8.61	8.77	8.06	8.77	4.84	1.71		
(**2**) I	9.69	8.24	8.92	8.54	8.28	8.60	2.98	7.75	7.75	7.31	7.31	8.00	8.40	7.34	7.52	7.34	4.45	1.45		
II	7.21	6.97	7.76	9.31	7.85	8.70	2.74	7.76	7.76	7.30	7.30	8.00	8.40	7.34	7.52	7.34	4.97	1.52		
(**5**) I	10.23	8.14	8.63	9.77	8.22	8.65	2.99	7.64	7.64	7.06	7.06	7.30	7.92	7.48	6.78	6.98			4.72	3.40	7.45	n.a.
II	9.51	7.95	8.52	9.96	7.87	8.51	2.40	7.59	7.59	7.26	7.26	7.01	7.71	7.45	7.19	6.93			4.78	3.44	7.45	n.a.
(**6**) I	9.60	8.01	8.23	8.77	8.02	n.a.	2.96	7.70	7.70	7.20	7.20	7.00	7.40	7.19	n.a.	7.19			4.66	3.38	n.a.	n.a.
II	9.18	8.00	8.18	8.83	n.a.	n.a.	2.45	7.71	7.71	7.22	7.22	7.02	7.26	7.16	n.a.	7.16			4.60	3.36	n.a.	n.a.

n.a. = Not assigned. Mebpy-ala = 4-COAlaCONH$_2$-4′-Mebpy; Mebpy-trpt = 4-COTrptCONH$_2$-4′-Mebpy.

in Fig. 2A. At a ratio $r = 0.1$ complex (**1**) causes a significant decrease of the characteristic CD band at 278 nm and an irrelevant alteration of the negative band at 245 nm. Increasing the ratio from $r = 0.3$ to $r = 0.5$ the intensity of the band at 278 nm further increases, while the intensity of the negative band at 245 nm remains practically unaffected. This behavior is similar to that observed in the case of interactions of the complex *cis*-(Cl,Cl)-[$RuCl_2$(NO)(terpy)]Cl with CT-DNA, which coordinates to the DNA bases without affecting the DNA helicity, remaining in B-type [6]. On the other hand the CD spectral changes caused by the complex (**2**) are more pronounced (Fig. 2B). A decrease in the intensity of the positive Cotton band at 278 nm and a ratio increase was observed. At a ratio $r = 0.5$ a new positive band appears at 323 nm, simultaneously with an increase of the intensity in the band at 245 nm indicating a strong perturbation of the DNA helix. The DNA conformation changes, probably reflect an additional interaction with the conjugated trp moiety of the complex. Similar CD spectral changes have been observed in the case where the complex [Ru(terpy)(4-CO_2-gly-L-his-L-lysCONH$_2$-4′-Mebpy)Cl]$^{3+}$ interacts with CT-DNA at the ratio $r = 0.5$ [16].

Complexes (**5**) and (**6**) cause insignificant CD spectral changes upon addition to CT-DNA in several ratios. In general the complexes affect only slightly the characteristic CD bands of B-type DNA, depending on the ratio. At ratio $r = 0.5$ the intensity of the positive band at 278 nm decreases about 10%, while the band at 245 nm remains practically unaffected in both cases. Similar changes have been associated with a dehydration process of the DNA and a shortening of the distance between the bottom of the major groove and the phosphate groups, causing by alkaline-earth metal cations [43], or the triply positive charged cation $[Co^{III}(NH_3)_6]^{3+}$ [44].

A comparison of the interactions of the chlorido complexes with the corresponding nitro ones would lead to the conclusion that the coordination binding of the ruthenium to the DNA bases provides the ability for an additional interaction of the amino acid moiety with the DNA. This interaction causes significant perturbation of the DNA helix. The nitro complexes interact rather slightly with the DNA helix causes only minor spectral changes. Fig. 2 presents the CD spectra of the DNA upon addition of the complexes studied.

^{23}Na NMR spectroscopy was used to study the local Na^+ exchange and the complexes in the surrounding area of the DNA helix. Both chlorido complexes (**1**) and (**2**) cause narrowing of the ^{23}Na signal from 23 Hz to 9 Hz and 10 Hz, respectively, upon increasing the ratio from $r = 0.1$ to $r = 0.5$, indicating an electrostatic interaction between the positively charged complexes and the DNA phosphates (Fig. 3(i)). This interaction causes an irreversible modification of the DNA polyanion, replacing the sodium cations and it is similar with that observed at the cases of *cis*-$PtCl_2(NH_3)_2$ and *trans*-$PtCl_2(NH_3)_2$ [45]. However, in the case of complexes (**5**) and (**6**), at the same ratios

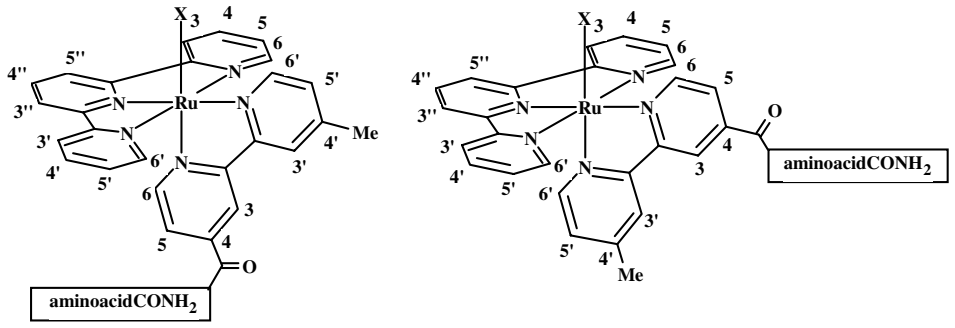

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Fig. 1. Structures of the positional isomers of the ruthenium oligopyridine complexes with conjugated amino acids and used atom numbering. Where aminoacidCONH$_2$ is AlaCONH$_2$, X = Cl (**1**), NO (**3**), NO$_2$ (**5**) and aminoacidCONH$_2$ is TrpCONH$_2$, X = Cl (**2**), NO (**4**), NO$_2$ (**6**).

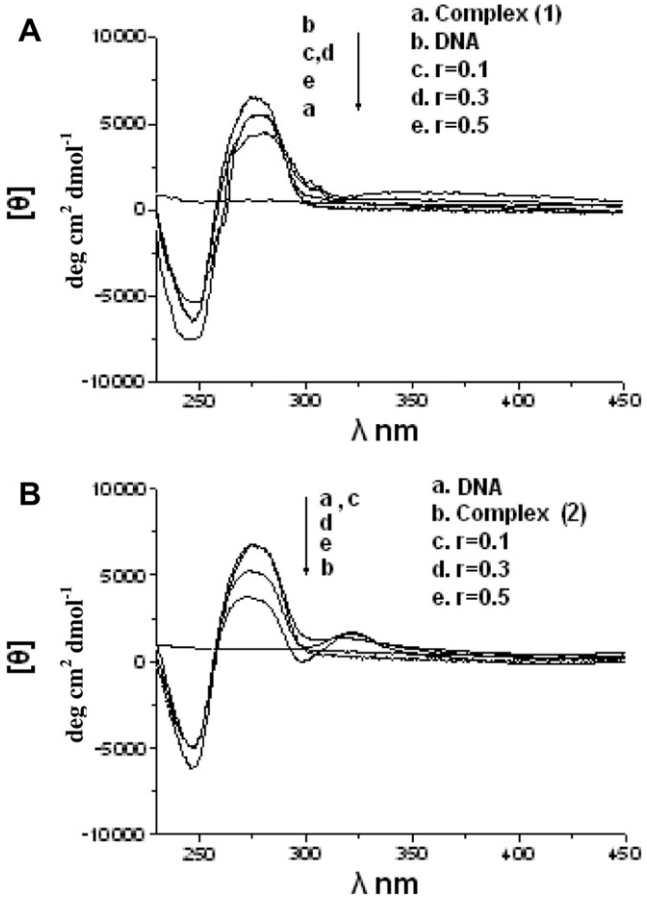

Fig. 2. Circular dichroism spectral changes of calf thymus DNA (70–300 bp) upon addition of complexes **(1)** and **(2)** (A and B, respectively) at ratios, r = [Ru]/[DNA], 0.1, 0.3 and 0.5. (DNA concentration 100 μM, T = 25 °C, pH 7.01).

($r = 0.5$), the narrowing of the ^{23}Na signal is smaller (13 and 12 Hz, respectively), probably due to their lower positive charge.

The ^{31}P NMR spectrum of the DNA, exhibits a narrow ($\Delta\nu_{1/2} = 76$ Hz) signal corresponding to phosphorous nuclei of the phosphates. Upon addition of the chlorido complexes (**1**) and (**2**) new peaks appear downfield to the initial ^{31}P signal, at 2.03 and 2.12 ppm correspondingly (Fig. 3(ii)). The new signals are consistent with unwinding of the DNA helix and distortion of the phosphate backbone [46]. A similar downfield shift was observed in the case of the complex [Ru(terpy)(4-CO_2H-4′-Mebpy-Gly-L-His-L-Lys-$CONH_2$)Cl](PF$_6$) with a value of 1.4 ppm, corresponding with coordination to DNA [16]. These differences observed in the ^{31}P signal, indicate that the nature of the conjugated moiety to the 4-CO_2H-4′-Mebpy-ligand, affect the binding of the complex, probably through an additional interaction with the DNA helix. Also, the complexes (**4**) and (**5**) cause – even higher – downfield shifts (3.88 and 3.90 ppm, respectively). Since the nitro complexes are not expected to coordinate with the DNA this effect can be explained by a change in the hydration sphere of the DNA close to the phosphates. This observation is consistent with that observed from the corresponding CD spectra. Fig. 3 presents the ^{23}Na and ^{31}P NMR spectra of the sonicated DNA following the addition of the complexes studied.

The intrinsic binding constants of complexes (**5**) and (**6**) with CT-DNA were determined from the decay of the absorbance at 474 and 490 nm, respectively, according to the reported procedure [47]. The K_b values were calculated about 0.8×10^3 M^{-1} and 1.8×10^3 M^{-1} in aqueous solution buffer (Tris–HCl 5 mM, NaCl 30 mM) at 25 °C for complexes (**5**) and (**6**) correspondingly. These values are about four orders of magnitude lower from those observed in cases of intercalative complexes (e.g. [Ru(bpy)$_2$(dppz)]$^{2+}$ (>10^6 M^{-1}) [48], [Ru(ip)$_2$(dppz)]$^{2+}$ (2.1×10^7) [49]) and are comparable with those of ruthenium complexes with bpy and phen ligands (e.g. [Ru(bpy)$_3$]$^{2+}$ (0.7×10^3 M^{-1}) [50], [Ru(5-NO_2-phen)$_3$]$^{2+}$ (1.0×10^3 M^{-1}) [50], [Ru(bpy)$_2$-(DIP)]$^{2+}$ (2.5×10^3 M^{-1}) [50]). The small difference of the DNA binding affinity between complexes (**5**) and (**6**) indicates that probably, the nature of the conjugated amino acid affects the binding mode. In general, the observed K_b values for both complexes (**5**) and (**6**), are consistent with a non-selective binding and are somewhat higher for a simple electrostatic binding of the complexes to DNA.

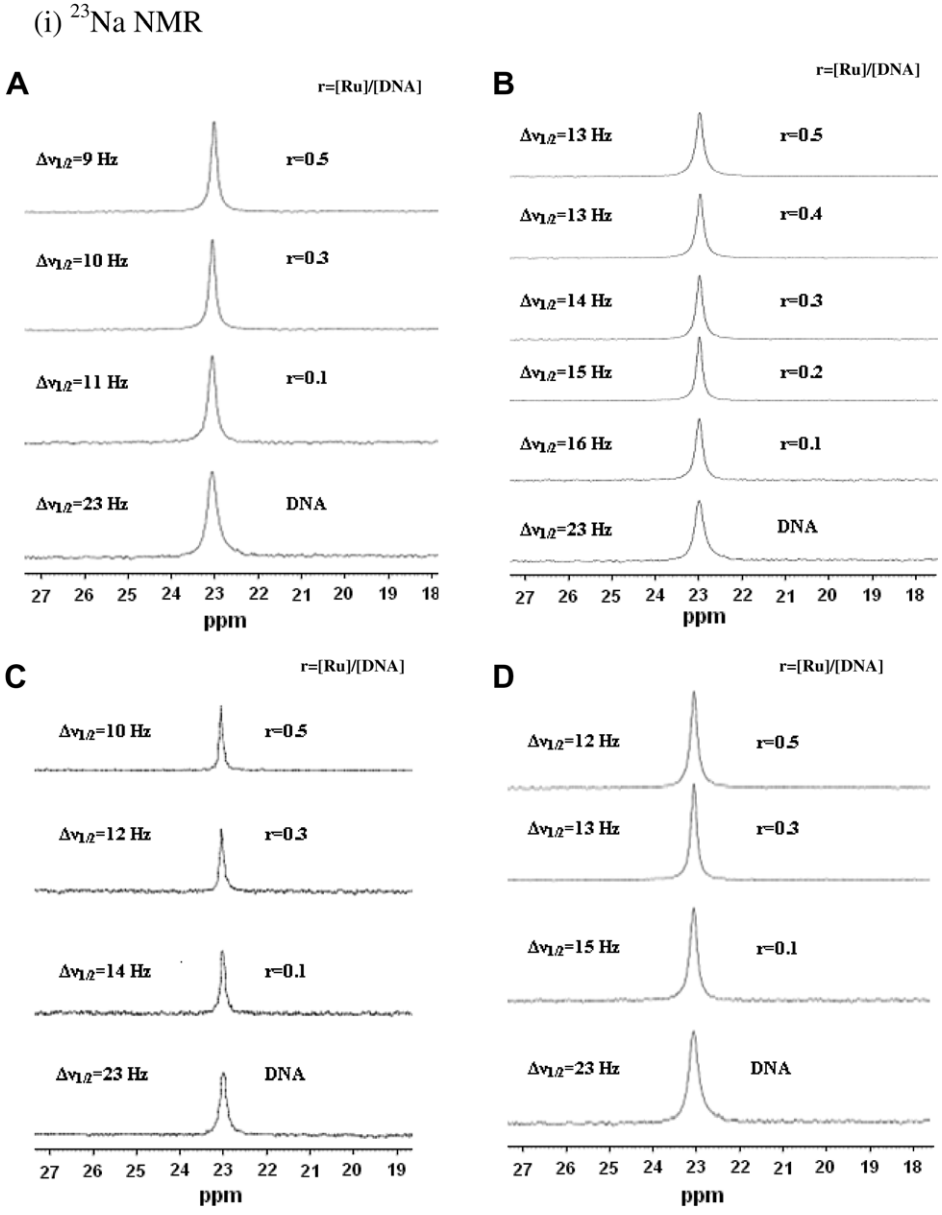

Fig. 3. (i) ^{23}Na NMR spectral changes of calf thymus DNA (70–300 bp) upon addition of complexes **(1)** (A), **(2)** (B), **(5)** (C), **(6)** (D), at increasing ratios (r = [Ru]/[DNA]) and (ii) ^{31}P NMR spectral changes of the same mixtures; **(1)** (E), **(2)** (F), **(3)** G, **(4)** H at ratios r = [Ru]/[DNA], 0.1–0.5 (DNA concentration = 6 mM, T = 298 K, pH 7.01).

The denaturation temperature of CT-DNA was determined in the absence and presence of the complexes (**1**), (**2**), (**5**) and (**6**). Complex (**1**) shows a ΔT_m (T_m(Ru) − T_m) value of −4 °C. This value indicates a double helix stabilization [51] and is similar to that observed for the complex $[RuCl(NH_3)_5]^{2+}$ ($\Delta T_m = -2$ °C) which binds coordinatively to DNA bases [52]. However, complex (**2**) causes an increase of the DNA denaturation temperature at about +5 °C. Since it is well known that increasing of the DNA denaturation temperature caused by intercalative molecules [51], it seems to be assumed that this positive value of $\Delta T_m = +5$ °C, reflects an average effect between the coordinatively binding of the complex to DNA bases and a DNA helix destabilization probably caused by the trp side chain. Modest values of ΔT_m were observed at the cases of complexes (**5**) and (**6**) ($\Delta T_m = -1$ °C and +1 °C, respectively), consistent with a solely binding of the complexes to the DNA.

4. Concluding remarks

In conclusion chlorido complexes (**1**) and (**2**) interact with DNA bases by coordination, while the conjugate amino acid provides additional interaction with the DNA helix. Electrostatic interactions causing irreversible modification of the DNA polyanion were also observed. The nature of the conjugated amino acid affects the binding of the complexes to DNA, since the aromatic nature of the trp

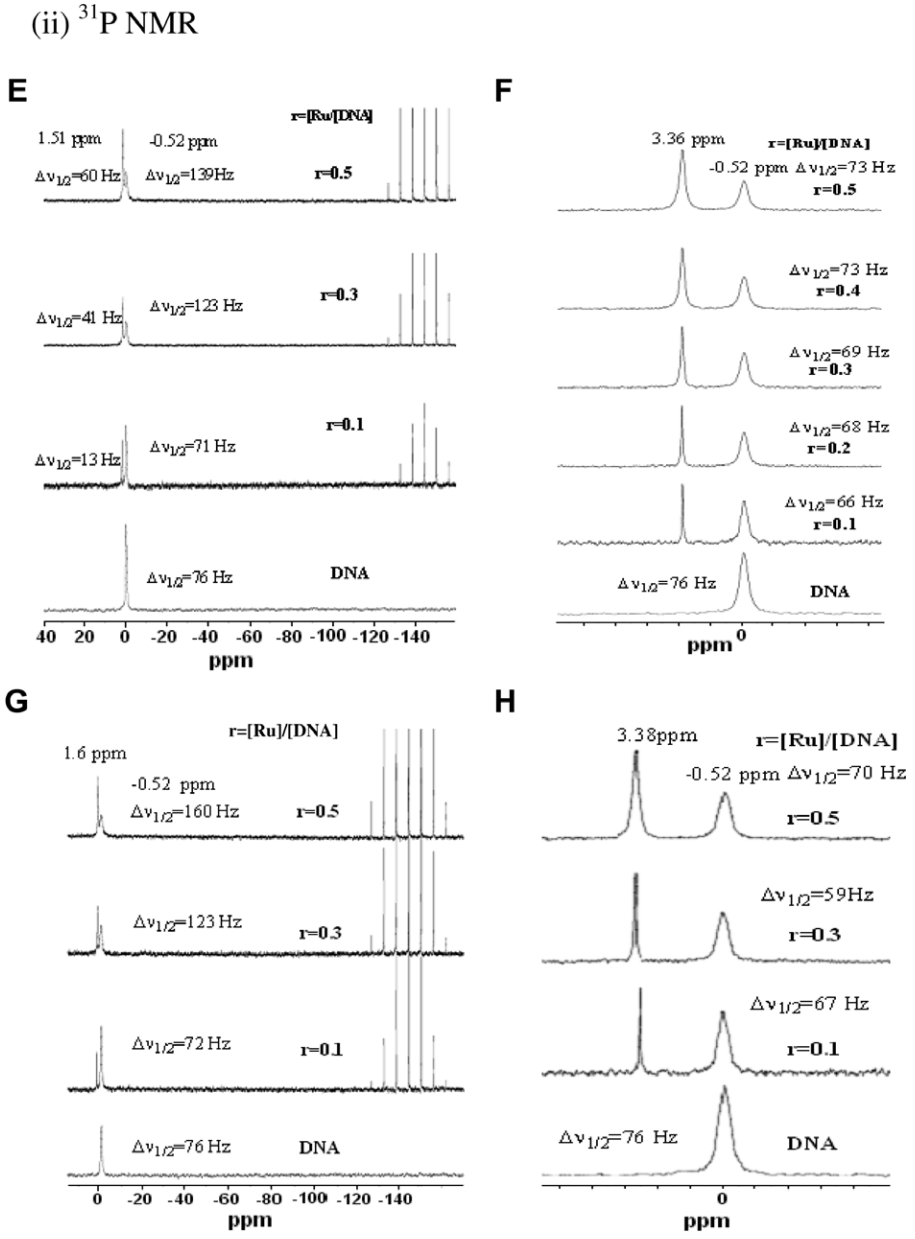

Fig. 3 (*continued*)

moiety of complex (**2**), causes important perturbations to the DNA helix. However, the changes caused by the nitro complexes (**5**) and (**6**) to the DNA helix are insignificant affecting only the ^{31}P NMR signal probably due to changes in the hydration sphere of the DNA, close to the phosphates.

5. Abbreviations

PyBOP benzotriazol-1-yl-oxytris(pyrrolidino)phosphonium hexafluorophosphate
DIPEA diisopropylethylamine
NMP *N*-methyl-pyrrolidone
DIP 4,7-diphyenylphenanthroline
Ip imidazole[4,5-*f*][1,10]phenanthroline
dppz dipyrido[3,2-α:2′3′-*c*]phenazine
Tris–HCl 2-amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride
PDT photodynamic therapy
Bpy 2,2′-bipyridine
Phen 1,10-phenanthroline
Terpy 2,2′:6′,2″-terpyridine
Mebpy 4́-methyl-2,2′-bipyridine
Fmoc 9-fluorenylmethyloxycarbonyl group
Boc *t*-butyloxycarbonyl group
Trt triphenylmethyl group
TMS tetramethylsilane
DMF dimethylformamide
ESI-MS electrospray ionization mass spectrometry
MLCT metal ligand charge transfer

TOCSY total correlation spectroscopy
CT-DNA calf thymus DNA

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