DNA Interactions of the Functionalized (Mixed Polypyridine)ruthenium(II) Complex Bis(2,2′-bipyridine-κN^1,$\kappa N^{1'}$)(methyl dipyrido[3,2-*a*:2′,3′-*c*]phenazine-11-carboxylate-κN^4,κN^5)ruthenium(2+) ($[Ru(bpy)_2(dppz\text{-}11\text{-}CO_2Me)]^{2+}$)

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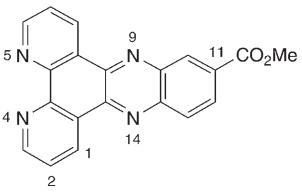
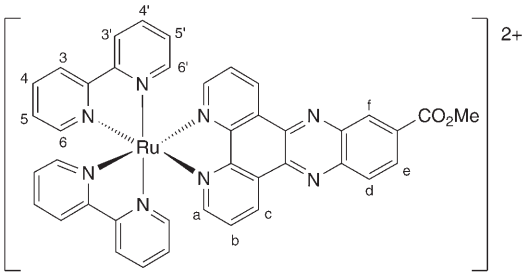
A novel polypyridine ligand, dipyrido[3,2-*a*:2′,3′-*c*]phenazine-11-carboxylic acid methyl ester (=dppz-11-CO_2Me), and its ruthenium(II) complex, $[Ru(bpy)_2(dppz\text{-}11\text{-}CO_2Me)]^{2+}$ (**1**), were synthesized and characterized. The binding properties of this complex to calf-thymus DNA (CT-DNA) were investigated by different spectrophotometric methods and viscosity measurements. The results suggest that the complex binds to DNA in an intercalative mode and serves as a molecular 'light switch' for DNA. When irradiated at 365 nm, the complex **1** promoted the photocleavage of plasmid pBR-322 DNA.

Introduction. – During the past decades, a number of transition-metal complexes have been utilized in the design and development of synthetic restriction enzymes, chemotherapeutic drugs, DNA footprinting agents, and stereoselective probes of nucleic acid structures [1–10]. Binding studies of small molecules to DNA are very important in the development of DNA molecular probes and new therapeutic reagents [11–13]. (Polypyridine)ruthenium(II) complexes can bind to DNA in a noncovalent-interaction fashion such as electrostatic binding, groove binding [14], intercalative binding, and partial intercalative binding [15]. Many useful applications of these complexes require that the complexes bind to DNA through an intercalative mode. Therefore, much work has been done on modifying the intercalative ligand. Of these, Ru^{II} complexes containing the ligand dppz (=dipyrido[3,2-*a*:2′,3′-*c*]phenazine) have emerged as the most promising metal-based molecular probes of DNA [16–18]. $[Ru(bpy)_2(dppz)]^{2+}$ (bpy=bipyridine) and $[Ru(phen)_2(dppz)]^{2+}$ (phen=1,10-phenanthroline) show no photoluminescence in aqueous solution at room temperature but luminesce brightly upon binding intercalatively with the dppz ligand between adjacent DNA base pairs, displaying the characteristic of molecular 'light switches'. Numerous other structural analogues with different shapes and electronic properties based on the $[Ru(phen)_3]^{2+}$ prototype have been synthesized and investigated [19–25]. However, the background luminescence of the free complexes in aqueous solution and their relatively weak binding constants represent limiting factors for their application as nonradioactive nucleic acid probes.

To find a sensitive luminescence probe for DNA, it is necessary to carry out a systematic study of the interactions of (polypyridine)ruthenium(II) complexes with DNA. We report herein the synthesis and characterization of a new polypyridine ligand, dipyrido[3,2-*a*:2′,3′-*c*]phenazine-11-carboxylic acid methyl ester (=dppz-11-CO_2Me), and of its Ru^{II} complex $[Ru(bpy)_2(dppz\text{-}11\text{-}CO_2Me)]^{2+}$ (**1**). Complex **1** was found to intercalate between the DNA base pairs and to be a highly sensitive luminescent sensor for double-strand DNA. Moreover, the complex promoted the photocleavage of pBR 322 under irradiation at 365 nm. We hope that our results will contribute to the understanding of DNA recognition and binding by Ru^{II} complexes, as well as to the rational design of new photoprobes and photonucleases for DNA.



dppz-11-CO_2Me



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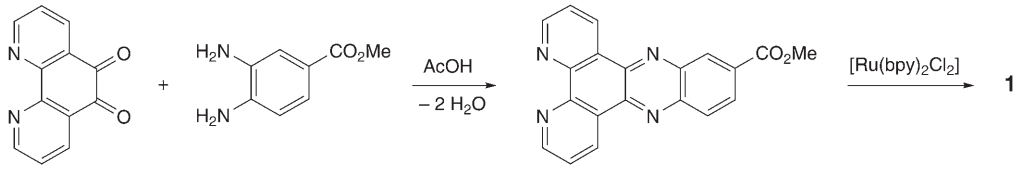
Results and Discussion. – 1. *Synthesis and Characterization.* The ligand dppz-11-CO_2Me was prepared by condensation of 1,10-phenanthroline-5,6-dione with methyl 3,4-diaminobenzoate on the basis of the method developed for the synthesis of the dppz ring system [26] (*Scheme*). $[Ru(bpy)_2(dppz\text{-}11\text{-}CO_2Me)]^{2+}$ (**1**) was then obtained in relatively high yield by direct reaction of dppz-11-CO_2Me with the appropriate mol ratio of *cis*-$[Ru(bpy)_2Cl_2]\cdot 2H_2O$ in ethylene glycol. The desired Ru^{II} complex was isolated as its perchlorate and was purified by column chromatography. In the ESI-MS of **1**, the ions $[M-ClO_4]^+$, $[M-2ClO_4-H]^+$, and $[M-2ClO_4]^{2+}$ were observed, and the determined m/z were consistent with the expected values.

Scheme. *Syntheses of the Ligand dppz-11-CO_2Me and of its Ru^{II} Complex* **1**



dppz-11-CO_2Me

Complex **1** gave well-resolved ^{1}H-NMR spectra (*Fig. 1*), permitting unambiguous identification and assessment of purity. The ^{1}H-NMR chemical shifts were assigned on the basis of ^{1}H,^{1}H-COSY experiments, and by comparison with the values of similar compounds [15–22]. Due to the shielding influences of the adjacent dppz-11-CO_2Me

and bpy moieties, the two halves of each bpy are chemically and magnetically nonequivalent, leading to eight signals corresponding to the bpy H-atoms: one set of four is associated with the half of bpy near the dppz-11-CO_2Me, the other set of four is associated with the half of bpy near the other bpy. Since the shielding effect of dppz-11-CO_2Me is obviously greater than that of bpy, the chemical shifts of the latter H-atoms occur more downfield than those of the former.

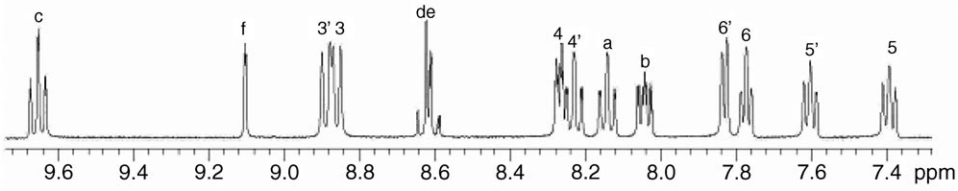


Fig. 1. *Aromatic region of the ^{1}H-NMR spectrum ((D$_6$)DMSO, 400 MHz) of complex* **1**

The UV/VIS absorption spectra of **1** showed three well-resolved bands in the range of 200 – 600 nm, characterized by intense $\pi \rightarrow \pi^*$ ligand transitions in the UV, as well as by a metal-to-ligand-charge-transfer (MLCT) transition in the VIS. The broad MLCT absorption band appears at 442 nm for complex **1**, which is attributed to Ru(dπ) $\rightarrow$ dppz-11-CO_2Me (π^*) transitions.

2. *DNA Binding.* 2.1. *UV/VIS Titration.* The application of electronic-absorption spectroscopy in DNA-binding studies is one of the most useful techniques [24]. Complex binding with DNA through intercalation usually results in hypochromism and bathochromism, due to the intercalative mode involving a strong stacking interaction between an aromatic chromophore and the base pairs of DNA. The extent of the hypochromism commonly parallels the intercalative binding strength. The absorption spectra of complex **1** (at a constant complex concentration) in the absence and presence of calf-thymus DNA (CF-DNA) are given in *Fig. 2*. As the concentration of DNA increased, the hypochromism in the IL band reached *ca.* 27.4% at 285 nm with a 3 nm red shift at a ratio [DNA]/[Ru] of 4.32. The MLCT band at 442 nm showed hypochromism of *ca.* 27.4% and a red shift of 3 nm under the same experimental conditions. Comparing the hypochromism of the complex with that of its parent complex $[Ru(phen)_3]^{2+}$ (hypochromism in the MLCT band at 445 nm of 12% and a red shift of 2 nm) [6], which interacted with DNA in a semi-intercalation or quasi-intercalation mode [25], and considering that the absorption spectrum of $[Ru(bpy)_3]^{2+}$, a typical electrostatic-binding complex, did not change upon the addition of the DNA [11], the spectral characteristics observed for complex **1** obviously suggest that **1** interacts with DNA most likely in a mode that involves a stacking interaction between the aromatic chromophore and the base pairs of DNA.

To analyze quantitatively the binding strength of complex **1**, the intrinsic binding constant K_b of **1** with DNA was obtained by intense $\pi \rightarrow \pi^*$ ligand transitions in the absorbance at 285 nm of **1** with increasing concentration of DNA and by using *Eqn. 1* [26], where [DNA] is the concentration of DNA (in base pairs), and ε_a, ε_f, and ε_b are the extinction coefficients of the apparent, free, and bound metal complex, respectively.

$$[DNA]/(\varepsilon_a - \varepsilon_f) = [DNA]/(\varepsilon_b - \varepsilon_f) + 1/[K_b(\varepsilon_b - \varepsilon_f)] \quad (1)$$

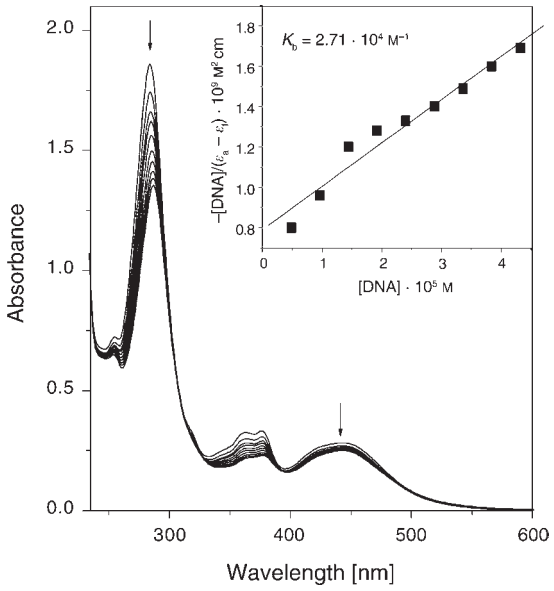


Fig. 2. *UV/VIS Absorption spectra of complex* **1** *in aqueous* Tris·*HCl buffer upon addition of CT-DNA*. [Ru] = $2 \cdot 10^{-5}$ M, [DNA] = $0 - 9.6 \cdot 10^{-5}$ M. Arrow shows the absorbance changes upon increasing DNA concentration. Inset: plots of $-10^9 \cdot [\mathrm{DNA}]/(\varepsilon_a - \varepsilon_f)$ (in M^2 cm) *vs.* [DNA] (in M) for the titration of DNA with the complex for the determination of the binding constant K_b.

When plotting $[\mathrm{DNA}]/(\varepsilon_a - \varepsilon_f)$ *vs.* [DNA], K_b is given by the ratio of the slope to the intercept, *i.e.*, K_b of complex **1** was determined as $2.71 \cdot 10^4$ M^{-1}. The experimental results suggest that upon modification of the dppz ligand, $[Ru(bpy)_2(dppz\text{-}11\text{-}CO_2Me)]^{2+}$ (**1**) preserves the small DNA binding affinity of its parent complex. The value is smaller than those of $[Ru(bpy)_2(dppz)]^{2+}$ (> 106 M^{-1}) [16], $[Ru(bpy)_2(dppx)]^{2+}$ (dppx = 7,8-dimethyldipyridophenazine; $8.8 \cdot 10^6$ M^{-1}) [12], $[Ru(phen)_2(phehat)]^{2+}$ (phehat = 1,10-phenanthrolino[5,6-*b*]1,4,5,8,9,12-hexaazatriphenylene; $2.5 \cdot 10^6$ M^{-1}) [19]. Furthermore, some other reported Ru^{II} complexes with extended, planar ligands, $[Ru(bpy)_2(dpq)]^{2+}$ (dpq = dipyrido[3,2-*d*:2′,3′-*f*]quinoxaline) and $[Ru(bpy)_2(dpqc)]^{2+}$ (dpqc = dipyrido-6,7,8,9-tetrahydrophenazine), show much smaller affinity with DNA ($5.9 \cdot 10^4$ M^{-1} and $8.5 \cdot 10^4$ M^{-1}) than $[Ru(bpy)_2(dppz)]^{2+}$ due to a smaller π-conjugated aromatic area [20]; $[Ru(bpy)_2(taptp)]^{2+}$ (taptp = 4,5,9,18-tetraazaphenanthreno[9,10-*b*]triphenylene) and $[Ru(bpy)_2(atatp)]^{2+}$ (atatp = acenaphtheno[1,2-*b*]-1,4,8,9-tetraazatriphenylene) also show smaller DNA affinity ($1.7 \cdot 10^5$ M^{-1} and $7.6 \cdot 10^4$ M^{-1}) than $[Ru(bpy)_2(dppz)]^{2+}$, the proposed reason is that the size of the ligands is too wide [22][23]. This data indicates that the size and the shape of the intercalated ligand has a significant effect on the strength of DNA binding, and the most suitable intercalating ligand leads to the highest affinity of complexes with DNA.

2.2. *The 'Light Switch' Effect of* $[Ru(bpy)_2(dppz\text{-}11\text{-}CO_2Me)]^{2+}$ *with CT-DNA.* Many (polypyridine)ruthenium(II) complexes luminesce intensively in the presence of DNA, but their strong background luminescence in the free form prevents them from

being a molecular 'light switch' for DNA. Reduction of the background luminescence might be achieved by introducing more heteroatoms into the structure of complexes. In the absence of DNA, complex **1** emits no luminescence in *Tris* buffer at room temperature but emits in organic solvents such as MeCN, DMSO, and MeOH (*Fig. 3*). The results suggest that the more polar the solvent is, the smaller relative intensities are observed. This phenomenon has also been found with the $[Ru^{II}(dppz)]$ complexes under similar conditions [13].

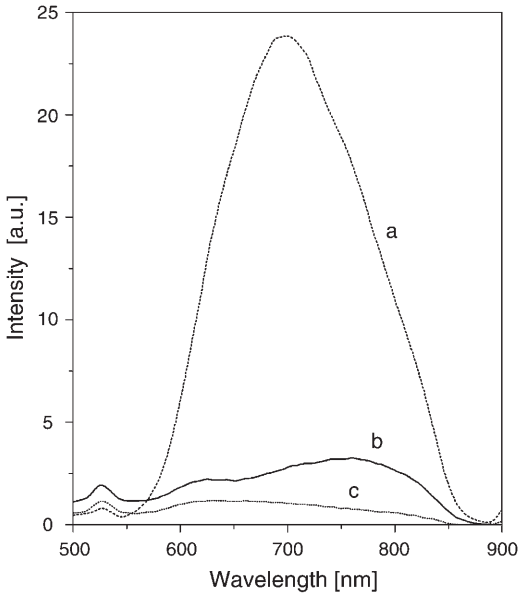


Fig. 3. *Fluorescence spectra of complex* ***1*** *in different solvents such as MeCN* (*a*), *DMSO* (*b*), *and MeOH* (*c*)

Fig. 4 shows the steady-state emission spectra of complex **1** in 5 mM *Tris*·HCl and 50 mM NaCl buffer (pH 7.2) in the absence and presence of CT-DNA. In the absence of DNA, complex **1** shows negligible luminescence in buffer at room temperature, with a maximum appearing at 633 nm. Upon addition of CT-DNA, however, luminescence increased, and the emission intensities of complex **1** increased by a factor of *ca.* 15.56, displaying the 'light-switch' behavior of complex **1**. The mechanism of the 'light-switch' effect has been studied intensively for $[Ru(bpy)_2(dppz)]^{2+}$, and all evidence points to H-bonding and/or excited-state H-atom transfer to the phenazine N-atoms as the mechanism of deactivation of the complexes' excited state [13][17][27–30]. Accordingly, a similar mechanism may be envisioned in our case. Data from the emission titration also implies that the complex can strongly interact with DNA and be protected by DNA efficiently since the hydrophobic environment inside the DNA helix reduces the accessibility of solvent H_2O molecules to the complex, and the complex mobility is restricted at the binding site, leading to a decrease of the vibrational modes of relaxation.

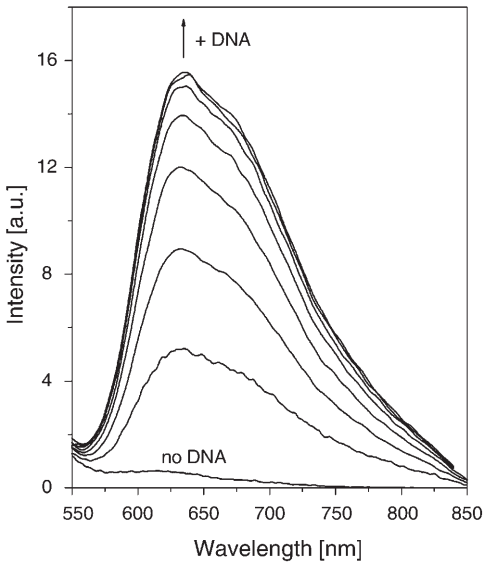


Fig. 4. *Fluorescence spectra of* **1** *in aqeous* Tris · *HCl buffer at 298 K in the presence of CT-DNA*. Arrow shows the intensity changes upon increasing DNA concentrations. $[Ru] = 2 \cdot 10^{-6}$ M, for complex [DNA]/[Ru] = 67.2 for **1**.

2.3. *Viscosity Measurements.* Hydrodynamic measurements that are sensitive to length changes, as reflected in viscosity and sedimentation, are regarded as the least-ambiguous and the most-critical tests of a binding model in solution, in the absence of crystallographic data [31][32]. A classical intercalation model demands that the DNA helix must lengthen as base pairs are separated to accommodate the binding ligand, which, in turn, leads to an increase of the viscosity of DNA [31][32]. The effect of added complex **1**, $[Ru(bpy)_3]^{3+}$, ethidium bromide (EB), or $[Ru(bpy)_2(dppz)]^{2+}$ on the viscosity of rod-like DNA are shown in *Fig. 5.* EB is well known to bind to DNA in the intercalation mode. Complex $[Ru(bpy)_3]^{2+}$, known to bind to DNA in the electrostatic mode, exerts essentially no effect on DNA viscosity. By contrast, upon increasing amounts of added **1**, the relative viscosity of DNA increased steadily, similarly to the effect of EB. Thus the increased degree of viscosity, which may depend on the affinity of a binding ligand to DNA, follows the order $EB > [Ru(bpy)_2(dppz)]^{2+} > \mathbf{1} > [Ru(bpy)_3]^{2+}$ and suggests that complex **1** binds to DNA in the intercalation mode. The incorporation of the CO_2Me group into the dppz ligand may cause steric hindrance when complex **1** interacts with DNA. Therefore, $[Ru(bpy)_2(dppz)]$ could intercalate more deeply and tightly into adjacent DNA base pairs than **1**.

2.4. *Enantioselective Binding.* Equilibrium-dialysis experiments offer the opportunity to examine the enantioselectivity of complexes binding to DNA. According to the proposed binding model by *Barton* and co-workers [33], the Δ enantiomer of the complex, a right-handed propeller-like structure, displays a greater affinity than the Λ enantiomer with the right-handed CT-DNA helix due to the appropriate steric

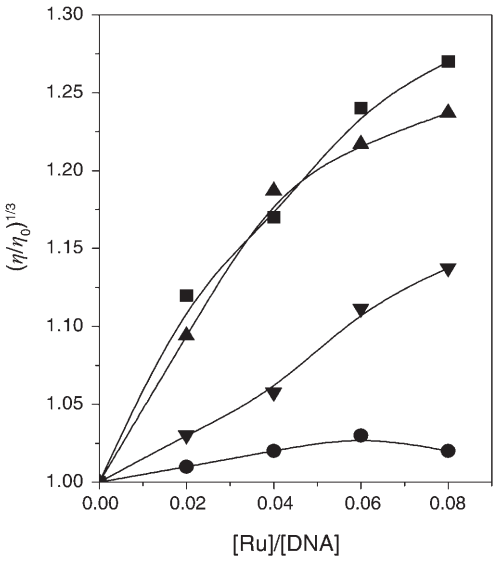


Fig. 5. *Effect of increasing amounts of EB* (■), *[Ru(bpy)$_3$]$^{2+}$* (●), **1** (▼), *and [Ru(bpy)$_2$(dppz)]$^{2+}$* (▲) *on the relative viscosity of CT-DNA*. Total DNA concentration 0.5 mM, *T* 30 ± 0.1°.

matching. This discrimination can be observed *via* equilibrium-dialysis experiments and provide strong evidence in support of interaction.

The CD spectra in the UV region of the dialysate of complex **1** shows a positive peak at 279 nm and a negative peak at 292 nm (*Fig. 6*). Although complex **1** has not been resolved into its pure enantiomers, and although we cannot determine which enantiomer of **1** enantioselectively binds DNA, it is certain that the complex could interact with CT-DNA enantioselectively.

2.5. *Photoactivated Cleavage of pBR-322 DNA by Ru^{II} Complex* **1**. The cleavage reaction on plasmid DNA can be monitored by agarose gel electrophoresis. When circular plasmid DNA is subjected to electrophoresis, relatively fast migration will be observed for the intact supercoil form (form I). If scission occurs on one strand (nicking), the supercoil will relax to generate a slower-moving open circular form (form II). If both strands are cleaved, a linear form (form III) that migrates between form I and form II will be generated [34]. As can be seen in *Fig. 7*, control photoreactions with DNA alone (*lane 0*) resulted in little or no DNA cleavage. By contrast, in the presence of increasing concentrations of complex **1** (*lanes 1–4*), the amount of form I of pBR-322 DNA diminished gradually, whereas form II increased. At the concentration of 25 μM (*lane 3*), **1** can almost promote the complete conversion of DNA from form I to form II, whereas at the concentration of 35 μM (*lane 4*), significant amounts of linear DNA (form III) are visible. Although DNA photocleavage by [Ru(phen)$_3$]Cl$_2$ has been reported to involve an $^1O_2$-based mechanism [35], the nature of the reactive intermediates involved in the efficient DNA photocleavage by complex **1** observed here is not clear. More detailed studies are currently underway to clarify the cleavage mechanism.

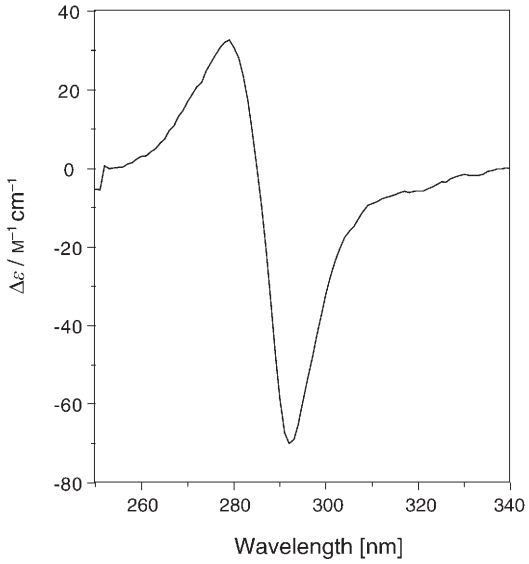


Fig. 6. *CD Spectrum of* **1** *after 42 h of dialysis against CT-DNA in a stirred aqueous solution*

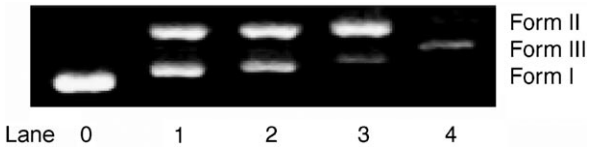


Fig. 7. *Photoactivated cleavage of pBR 322 DNA in the presence of* Ru^{II} *complex* **1**, *light after 60 min irradiation at 365 nm. Lane 0*, DNA alone; *lanes 1–4*, effect of different concentrations of complex **1**: (*lane 1* 5, *lane 2* 15, *lane 3* 25, and *lane 4* 35 μM) on DNA.

3. Conclusions. – A novel complex $[Ru(bpy)_2(dppz\text{-}11\text{-}CO_2Me)]^{2+}$ (**1**) was synthesized and characterized. The binding properties of this complex to CT-DNA were investigated by different spectrophotometric methods and viscosity measurements. The results suggest that the complex binds to DNA in an intercalative mode and serves as a molecular 'light switch' for DNA. When irradiated at 365 nm, the complex promoted the photocleavage of plasmid pBR-322 DNA.

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Experimental Part

General. All reagents and solvents were purchased commercially and used without further purification, unless noted otherwise. Doubly distilled H_2O was used to prepare buffers. CT-DNA was obtained from the *Sino-American Biotechnology Company*. The 1,10-phenanthroline-5,6-dione [36] and *cis*-$[Ru(bpy)_2Cl_2]\cdot 2\,H_2O$ [37] were prepared according to literature procedures. All other materials were

commercially available and reagent-grade. UV/VIS Spectra: *Perkin-Elmer Lambda-25* apparatus; λ_{max} in nm (ε in $dm^3\ mol^{-1}\ cm^{-1}$). Fluorescence spectra: *Perkin-Elmer LS-55* spectrophotometer; at r.t. CD Spectra: *Jasco J715* spectropolarimeter. ^{1}H-NMR Spectra: *Bruker Avance-400* apparatus; at 400 MHz in (D_6)DMSO at r.t.; δ in ppm rel. to Me_4Si, *J* in Hz. ESI-MS: *Finnigan MAT-LQC* system, with MeCN as mobile phase; spray voltage 4.50 KV, tube-lens offset 30.00 V, capillary voltage 23.00 V, and capillary temp. 200°; in *m*/*z*. Elemental analyses (C, H, N): *Perkin-Elmer 240Q* elemental analyzer.

*Dipyrido [3,2-*a*:2′,3′-*c*]phenazine-11-carboxylic Acid Methyl Ester* (dppz-11-CO_2Me). A mixture of methyl 3,4-diaminobenzoate (220 mg, 1.32 mmol), 1,10-phenanthroline-5,6-dione (420 mg, 2.0 mmol), and glacial AcOH (20 ml) was heated under reflux with stirring for 2 h. The soln. was allowed to cool, filtered, diluted with H_2O, and neutralized with conc. aq. NH_3 soln. The resulting yellow precipitate was collected and purified by column chromatography (CC) (Al_2O_3, EtOH/toluene 5:1): dppz-11-CO_2Me (510 mg, 75%). Amorphous, dark yellow solid. Anal. calc. for $C_{20}H_{13}N_4O_3$: C 67.22, H 3.67, N 15.68; found: C 67.09, H 3.71, N 15.54.

Bis(2,2′-bipyridine-κN^{1},$\kappa N^{1'}$*)(methyl dipyrido[3,2-*a*,2′,3′-*c*]phenazine-11-carboxylate-*κN^{4},κN^{5}*)-ruthenium(2+) Perchlorate Hydrate (1:1:2)* [$Ru(bpy)_2$(dppz-11-CO_2Me)]$(ClO_4)_2 \cdot 2\,H_2O$; **1**). A mixture of *cis*-[$Ru(bpy)_2Cl_2] \cdot 2\,H_2O$ (130 g, 0.25 mmol), dppz-11-CO_2Me (85 mg, 0.25 mmol), and ethylene glycol (15 ml) was thoroughly deoxygenated. The purple mixture was heated for 8 h at 150° under Ar. When the soln. finally turned red, it was cooled to r.t., and an equal volume of sat. aq. $NaClO_4$ soln. was added under vigorous stirring. The red solid was collected and washed with small amounts of H_2O, EtOH, and Et_2O, dried under vacuum, and purified by CC (neutral Al_2O_3; MeCN/toluene 2:1): 150 mg (65%) of **1**. UV/VIS (MeCN): 441 (13000), 362 (14000), 282 (89500). ^{1}H-NMR (400 MHz, (D_6)DMSO): 9.66 (*dd*, *J* = 6.4, 1.6, 2 H); 9.10 (*s*, 1 H); 8.89 (*d*, *J* = 8, 2 H); 8.86 (*d*, *J* = 8, 2 H); 8.28–8.21 (*m*, 4 H); 8.15 (*d*, *J* = 8, 2 H); 8.12 (*d*, *J* = 1.6, 2 H); 8.04 (*m*, 2 H); 7.83 (*d*, *J* = 5.2, 2 H); 7.77 (*t*, 2 H); 7.60 (*t*, 2 H); 7.39 (*t*, 2 H); 3.82 (*d*, *J* = 1.6, 2 H). ESI-MS (MeCN): 870.5 ($[M - ClO_4]^+$), 770.7 ($[M - 2ClO_4]^+$), 385.6 ($[M - 2ClO_4]^{2+}$). Anal. calc. for $C_{40}H_{34}Cl_2N_8O_{13}Ru$: C 48.58, H 3.44, N 11.34; found: C 48.39, H 3.46, N 11.05.

UV/VIS Titrations. All experiments were carried out in buffer *A* (5 mM *Tris*·HCl, 50 mM NaCl, pH 7.2) at r.t. A soln. of CT-DNA in buffer *A* gave a ratio of UV absorbances at 260 and 280 nm of *ca.* 1.8:1 to 1.9:1, indicating that the CT-DNA was sufficiently free of protein [38]. The concentration of CT-DNA was determined spectrophotometrically ($\varepsilon_{260} = 6600\ M^{-1}\ cm^{-1}$) [39]. Stock solns. were stored at 4° and used within 4 d. Titration experiments were performed with a fixed Ru^{II} complex concentration (10 μM), to which the CT-DNA stock soln. was added up to a ratio [DNA]/[Ru] of 0.51:1. The complex/DNA solns. were allowed to equilibrate for 5 min before spectra were recorded.

Viscosity Measurements. These experiments were carried out with an *Ubbelodhe* viscometer maintained at 30.0 ± 0.1° in a thermostated bath. DNA samples of *ca.* 200 bp average length were prepared by sonication [40]. The flow time was measured with a digital stopwatch, and each sample was tested three times to get an average calculated time. Data were presented as $(\eta/\eta_0)^{1/3}$ *vs.* binding ratio [41], where η is the viscosity of DNA in the presence of the complex, η_0 being the viscosity of free DNA.

For the gel-electrophoresis experiments, supercoiled pBR-322 DNA (0.1 μg) was treated with Ru^{II} complex in 50 mM *Tris*·HCl and 18 mM NaCl buffer (pH 7.2). Then the soln (preincubated in the dark for 1 h) was irradiated for 60 min inside the sample chamber of the *Perkin-Elmer LS-55* spectrofluorimeter (λ_{ex} 365 ± 5 nm, slit width 5 nm). The sample was analyzed by electrophoresis for 30 min at 75 V in *Tris*·HOAc buffer on a 1% agarose gel. The gel was stained with 1 $\mu g/ml^{-1}$ EB and photographed under UV light.

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