Synthesis of dinitroxyl *cis*-diaminoplatinum(II) complexes and their interaction with DNA

V. D. Sen', A. V. Kulikov, A. V. Shugalii, and N. P. Konovalova*

Institute of Chemical Physics in Chernogolovka, Russian Academy of Sciences, 142432 Chernogolovka, Moscow Region, Russian Federation. Fax: +7 (096) 515 3588. E-mail: senvd@icp.ac.ru

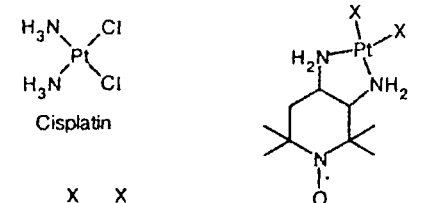
Dinitroxyl complexes of platinum, *cis*-$Pt^{II}(APO)_2X_2$, where APO is 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl, were obtained by either a direct reaction of APO with K_2PtX_4 (X = Cl or I) or a replacement of iodide ligands in *cis*-$Pt^{II}(APO)_2I_2$ by nitrate and oxalate ligands. The interaction of water-soluble *cis*-$Pt^{II}(APO)_2(NO_3)_2$ with ox spleen DNA resulted in platinated DNA with a degree of modification (r) ~7 times lower than that obtained with *cis*-$Pt^{II}(NH_3)_2Cl_2$ (cisplatin). Melting point T_m, melting range ΔT, and the degree of hyperchromicity ΔH for platinated DNA showed that for equal r values, the *cis*-$Pt^{II}(APO)_2$—DNA adducts increase heterogeneity in the DNA structure much more effectively than the *cis*-$Pt^{II}(NH_3)_2$—DNA adducts. Poor platinating activity, substantial disturbance of the DNA structure, as well as low toxicity and moderate antitumor activity of *cis*-$Pt^{II}(APO)_2X_2$ complexes are probably explained by steric hindrances caused by two bulky APO ligands.

Key words: platinum(II) complexes, nitroxyl radicals, DNA, platination, *cis*-diamminedichloroplatinum.

Platinum(II) complexes such as *cis*-diamminedichloroplatinum(II) (cisplatin) can add to DNA, thus disturbing its structure and the replication process *in vivo*. There is strong evidence[1] that the platination of DNA is the key reaction that determines the cytostatic properties of the amino complexes of platinum. Recently, we have synthesized new complexes of the general formula **1**, which contain the nitroxyl radical (*trans*-3,4-diamino-2,2,6,6-tetramethylpiperidine-1-oxyl (DAPO)[2]) as a diaminoligand, and studied their interaction with DNA in comparison with cisplatin.[3]

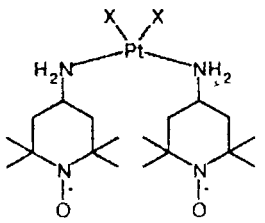
Earlier,[4,5] the synthesis of platinum diamino-complexes **2a—c** has been reported, but detailed procedures and elemental analysis data have not been given, which precludes rationalization of some discrepancies in the properties of complexes **2b,c** described[4,5] and obtained in the present work. The biological properties of complexes **2** and their reactivity with respect to DNA have not been reported.

In continuation of the study of aminonitroxyl complexes of platinum, we synthesized complexes **2a—d**, studied their structures, interaction with DNA, the properties of adducts that formed, and toxicity and antitumor activity.



Cisplatin

1



2a—d

a: X = Cl
b: X = I
c: X = NO_3
d: X + X = COOH, COOH

Experimental

The platinum content in complexes was determined by atomic-absorption spectroscopy on an AAS-3 spectrometer and by elemental analysis of the complexes from the residue weight (PtO_2) following combustion. The results of both determinations coincide within the measurement error (±3 rel. %). IR spectra (Vaseline oil) were recorded on Specord 75-IR (400—4000 cm^{-1}) and Specord M-82 (200—600 cm^{-1}) spectrometers. ESR spectra were obtained on an SE/X 2544 instrument at room temperature and 77 K (UHF power, 2 mW; modulation, 0.32 mT).

The initial **4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl** (APO) was obtained as described earlier[6] and purified by distillation *in vacuo* with a Vigreux distilling column 30 cm long; b.p. 90—93 °C (3 Torr).

***cis*-Dichlorobis(4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl)platinum(II) (2a).** A solution of 4-amino-2,2,6,6-tetramethylpiperidin-1-oxyl (342 mg, 2 mmol) in 2 mL of water

Translated from *Izvestiya Akademii Nauk. Seriya Khimicheskaya*, No. 8, pp. 1640—1644, August, 1998.

was added with stirring to a solution of K_2PtCl_4 (415 mg, 1 mmol) in 4 mL of water. The minor suspension that formed was removed by fast filtration through a dense glass filter. The solution was stirred for 2 h and then left at 10 °C for 12 h. The precipitate that formed was filtered off, washed successively with water, ethanol, and ether, and dried *in vacuo* ($1.5 \cdot 10^{-2}$ Torr) over P_2O_5. Complex **2a** was obtained (473 mg, 78%) as brick-yellow planar prisms, which decompose (darkening) without melting at ≥240 °C (*cf.* Ref. 4: m.p. 257—260 °C (decomp.)).

***cis*-Diiodobis(4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl)platinum(II) (2b).** Solutions of KI (730 mg, 4.4 mmol) in 5 mL of water and 4-amino-2,2,6,6-tetramethylpiperidin-1-oxyl (342 mg, 2 mmol) in 2 mL of water was successively added dropwise with stirring to a solution of K_2PtCl_4 (415 mg, 1 mmol) in 10 mL of water. Stirring was continued for 4 h. The yellow precipitate that formed was filtered off, washed successively with water, ethanol, and ether, and dried *in vacuo* ($1.5 \cdot 10^{-2}$ Torr). Complex **2b** was obtained (738 mg, 93%) as small yellow crystals. Crystallization from acetone gave orange prisms, which become turbid at ≥160 °C, then gradually darken, and melt at 252—254 °C (decomp.) (*cf.* Ref. 5: m.p. 244 °C). According to the IR spectrum, solvate acetone is present in crystals obtained (acetone could not be removed on drying *in vacuo* ($1.5 \cdot 10^{-2}$ Torr) at 56 °C for 1 h), and their elemental composition is best represented by the formula $\mathbf{2b} \cdot 0.5Me_2CO$.

***cis*-Dinitratobis(4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl)platinum(II) (2c).** A solution of $AgNO_3$ (680 mg, 4 mmol) in 10 mL of water was added to a suspension of complex **2b** (1.582 g, 2 mmol) in 40 mL of EtOH and stirred for 12 h on a magnetic stirrer in darkness. The precipitate of AgI that formed was thoroughly separated by centrifugation and filtration through a dense glass filter to give an orange solution of **2c**. The course of reaction was monitored by TLC on Silufol plates by following the consumption of the initial **2b** (R_f = 0.75) and accumulation of **2c** (R_f = 0.21, acetone—chloroform, 3 : 2). The completion of the reaction was also determined from the absence of immediate turbidity upon addition of a drop of 0.5 *M* KCl to three drops of the solution. The solution of **2c** obtained was concentrated *in vacuo* to ~5 mL, acetone (10 mL) added with stirring, and the reaction mixture left for 4 h to complete crystallization. The crystals were filtered off, washed with acetone (3×3 mL), and dried *in vacuo* to yield complex **2c** (802 mg, 59%). The product was crystallized from acetone and dried *in vacuo* at 56 °C for 1 h to give orange prismatic crystals, whose IR spectrum and elemental composition correlate well with the structure of monohydrate $\mathbf{2c} \cdot H_2O$. The crystals of $\mathbf{2c} \cdot H_2O$ become turbid at ≥170 °C, darken at ~195 °C (decompose), and do not melt below 250 °C (*cf.* Ref. 5: m.p. 135 °C).

***cis*-Oxalatobis(4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl)platinum(II) (2d).** A solution of $K_2C_2O_4 \cdot H_2O$ (550 mg) in 20 mL of water was added with stirring to a solution of complex **2c** (2 mmol), which was obtained as described above, in 50 mL of EtOH. The reaction mixture was stirred for 2 h and then left at 10 °C for 12 h. The pale rose crystals that formed were filtered off, washed with water and acetone, and dried *in vacuo* to give complex **2d** (887 mg, 71%). The crystals of **2d** darken without melting at ~250 °C.

Platination of DNA with complex 2c. Ox spleen DNA (Reakhim, Russia) was disintegrated by squeezing its solution (~1 mg mL^{-1}) through the needle of a medical syringe 0.1 mm in diameter. The average molecular weight of fragmented DNA was estimated by measuring the characteristic viscosity[7] and amounted to $\sim 1.65 \cdot 10^6$, or ~2500 nucleotide pairs. The DNA concentration was determined from the optical density of a sample at 260 nm, using the extinction coefficient $\varepsilon = 6.6 \cdot 10^3$ L mol^{-1} cm^{-1}. Fragmented DNA was modified with platinum complexes as follows. A reaction mixture containing DNA (the initial concentration, 0.5 g L^{-1}) and **2c** or cisplatin, was incubated in 0.01 *M* $NaHCO_3$ at 37 °C for 24 h. The initial ratio r_{in} = [complex]/[nucleotide] in the mixture was 0.04—0.3. The crystals of **2c** are poorly soluble in water (~0.1 mg mL^{-1} at 25 °C). However, an aqueous solution of **2c** with a concentration required for platination of DNA (~1—2 mg mL^{-1}) can be prepared by diluting its solution in EtOH (see procedure above) with water with subsequent removal of ethanol *in vacuo*. Modified DNA was separated from an excess of **2c** by dialysis against 0.01 *M* $NaHCO_3$ at 4 °C for 12 h with subsequent gel filtration on Sephadex G-50. The degree of modification r = [bound $Pt^{II}(APO)_2$]/[nucleotide] was determined following separation of the unconsumed complex. The amount of bound $Pt^{II}(APO)_2$ was found by ESR from the content of the >N—O· groups or by atomic-absorption spectroscopy from the platinum content. The number of the bound $Pt^{II}(APO)_2$ groups obtained by different methods coincided within the experimental errors (5—20%; a greater error corresponds to lower concentrations that are determined).

Melting point T_m, melting range ΔT, and the degree of hyperchromicity ΔH of platinated DNA were determined as described earlier.[3]

Determination of toxicity and antitumor activity. Complexes **2a—d** were introduced intraperitoneally into animals in the form of a suspension in a Tween-80—H_2O (1 : 9) mixture. Cisplatin was dissolved in water for injections. The overall toxicity (LD_{50}) of the compounds was determined for a single injection into BDF_1 mice. Antitumor activity was studied with respect to leukemia P 388. Leukemia was transinoculated intraperitoneally into BDF_1 mice with an inoculum containing 10^6 cells. The criterion of antileukemia activity of the complexes is the percentage of lifetime extension (LTE) of treated animals compared to nontreated ones: LTE = $[100(T/N - 1)]$, where T and N are the average lifetimes (in days) of treated and nontreated animals, respectively. Animals that remained alive at the end of experiment (60 days) were taken into account separately (Table 1).

Table 1. Toxicity and antileukemia (P 388) activity of complexes **2a—d** [a]

Complex	LD_{50}[b]/mg kg^{-1}	Dose/mg kg^{-1}	LTE[c] (%)
2a	570	190	106
$\mathbf{2b} \cdot 0.5Me_2CO$	140	46	0
$\mathbf{2c} \cdot H_2O$	500	166	79 (1/6)
2d	380	127	76
Cisplatin	12	4.0	140 (1/10)

[a] Compounds were introduced intraperitoneally into animals daily for 6 days.

[b] Dose causing the death of 50% of healthy mice.

[c] The average lifetime of animals in the untreated group was 11.2 days. The number of recovered animals (the lifetime >60 days) and the total number of animals in the group are given in parentheses as the numerator and the denominator, respectively.

Results and Discussion

Complexes **2a,b** were synthesized in an aqueous solution by a direct reaction of 4-amino-2,2,6,6-tetramethylpiperidin-1-oxyl with K_2PtCl_4 or K_2PtI_4, respectively, the latter being generated *in situ* from K_2PtCl_4 and KI. Synthesis of **2c** involved treatment of complex **2b** with a stoichiometric amount of $AgNO_3$. The course of the reaction was monitored by TLC by following the consumption of the initial **2b** and its completion evidenced from a negative test for the presence of residual $AgNO_3$ in the reaction mixture (see Experimental). After AgI that precipitated was thoroughly separated, **2c** was either isolated from its concentrated solution or treated with potassium oxalate to give **2d**.

Complexes **2a—d** are yellow or orange crystalline substances. The crystals of the complexes became opaque at 160—200 °C and darken (decompose) as temperature increases to 240—260 °C. All complexes are very poorly soluble in water (≤0.1 mg mL^{-1}). However, an aqueous solution of nitrate complex with the concentration of ~5 mg mL^{-1} can be obtained by partial evaporation of an aqueous—ethanol reaction solution *in vacuo* in the synthesis of **2c**. This feature of **2c** is probably explained by its tendency to form hydrate (see below). An oversaturated solution of **2c** thus obtained was used for the platination of DNA.

Data from elemental analysis (Table 2) show good agreement between the calculated and experimental values. It is known[8] that when K_2PtX_4 reacts with amines, the stronger *trans*-influence of halide ligands compared to N-ligands determines the *cis*-structure of the complexes formed. The high yield of **2d** (which cannot have the *trans*-structure because one of the angles in the five-membered chelate ring of the oxalate ligand cannot be equal to 180°) is an argument in favor of the *cis*-structure of the complexes obtained by us. The IR spectrum of complex **2a** exhibits a doublet of Pt—Cl absorption bands at 332 and 325 cm^{-1} characteristic of *cis*-complexes. These frequencies virtually coincide with those of a similar doublet in the spectrum of *cis*-$Pt^{II}(NH_3)_2Cl_2$ (330 and 323 cm^{-1}).[9] Taking into account the conclusion that the Pt—Cl stretching vibrations in *cis*-complexes do not depend on the structure of aminoligands,[9] the coincidence in ν(PtCl) frequencies is strong evidence of the *cis*-structure of **2a**.

In the IR spectra of **2a—d**, the bands at 3118—3245 and 1587—1607 cm^{-1} correspond to the stretching and deformation vibrations of the NH_2 groups, respectively. Recrystallization of **2b** from acetone leads to the appearance of a band of the carbonyl group in the IR spectrum of the product (1700 cm^{-1}). Such a result is explained by formation of a stable solvate. Based on the elemental analysis data, the structure **2b** · $0.5Me_2CO$ was attributed to this complex. Independent of the way in which nitrate **2c** was isolated and purified, its IR spectrum exhibited bands of water of crystallization (3370 and 3490 cm^{-1}). The structure of monohydrate **2c** · H_2O for this complex corresponds best to data from elemental analysis. These features of the structures of complexes **2b,c** have not been noted earlier.[5] In addition, compared to the published data,[5] **2c** · H_2O obtained by us decomposes without melting at a higher temperature and stays at the origin on TLC on silica gel in a $CHCl_3$—benzene system. The expected bands of the stretching vibrations of the carbonyl group of the oxalate ligand are present at 1674 and 1692 cm^{-1} in the IR spectrum of complex **2d**.

Three basic lines with a *g*-factor of 2.0056 and a splitting a_N of 1.68 mT on the nitrogen nucleus are observed in the ESR spectra of aqueous solutions of radicals **2a—d** at room temperature (Fig. 1). These values of *g*-factor and splitting are characteristic of aqueous solutions of six-membered nitroxyls. For all complexes, except **2b**, two additional lines are observed between the three basic ones, which are due to exchange interaction between the nitroxyl groups in the biradical.[10] The shape of the ESR spectra of **2a** and **2c** is close to that cited in the literature.[4,5] In the case of biradical **2d**, each of the additional spectral components is split, in turn, into two extra lines. This means[10] that the exchange interaction value $J < a$. In the case of biradical **2b**, no additional component is observed. Thus, the nature of ligands X substantially influences

Table 2. Elemental analysis and IR spectroscopic data of complexes **2a—d**

Compound	Found / Calculated (%)				Molecular formula	IR (Vaseline oil)	
	C	H	N	Pt		ν/cm^{-1}	Assignment
2a	35.25 35.53	6.07 6.29	9.44 9.21	33.0 32.1	$C_{18}H_{38}Cl_2N_4O_2Pt$	1587, 1607, 3118, 3178, 3200 332, 325	NH_2 PtCl
2b · $0.5Me_2CO$	28.65 28.55	5.35 5.93	6.93 6.83	22.9 23.8	$C_{18}H_{38}I_2N_4O_2Pt \cdot 0.5(CH_3)_2CO$	1602, 3120, 3180, 3197, 3245 1700	NH_2 Me_2CO
2c · H_2O	31.90 31.81	5.96 5.93	12.36 12.37	28.9 28.7	$C_{18}H_{38}N_6O_8Pt \cdot H_2O$	1600, 1633, 3128, 3210 973, 997, 1272, 1290, 1492, 1513 3370, 3490	NH_2 NO_3 H_2O
2d	38.16 38.40	5.98 6.12	9.17 8.96	29.7 31.2	$C_{20}H_{38}N_4O_6Pt$	1604, 3146, 3225 1674, 1692	NH_2 C=O

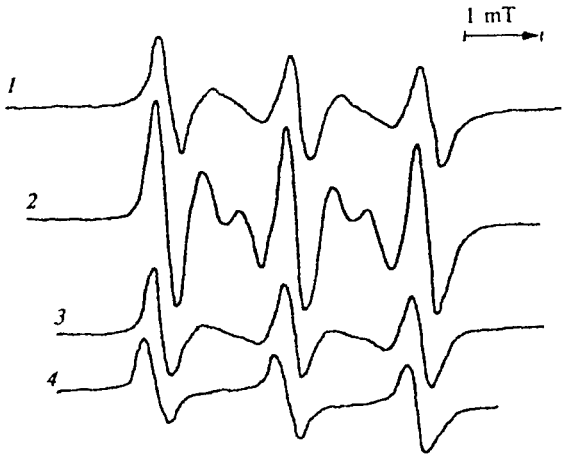


Fig. 1. ESR spectra of aqueous solutions of **2a—d** at room temperature: (*1*) **2c**, (*2*) **2d**, (*3*) **2a**, and (*4*) **2b**.

the parameters of exchange interaction between two nitroxyl groups.

The ESR spectrum of an ethanolic solution of biradical **2c** at 77 K (Fig. 2, curve *1*) is strongly distorted compared to that of monoradical because of a dipole-dipole magnetic interaction between the nitroxyl groups. The parameter d_1/d values (0.80), the second central moment (4.60 mT^2), and line splitting (1.55 mT) give[10] close values for the distance between the nitroxyl groups (1.16, 1.18, and 1.22 nm, respectively).

We studied platination of DNA with complex **2c** for which rather high concentration in water can be obtained (see Experimental). The platinating activity of the complexes was defined as the degree of modification r at the initial reagent ratio r_{in} = [complex]/[nucleotide] = 0.3. For **2c**, r = 0.019, which is ~7 times lower than that for cisplatin (r = 0.13). It is interesting that

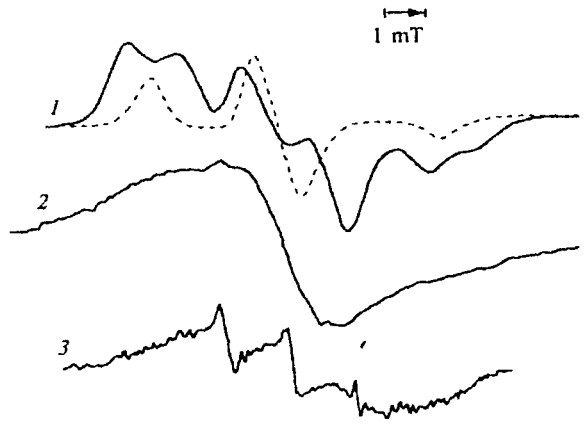


Fig. 2. ESR spectra of complex **2c** in ethanol at 77 K (*1*) (for comparison, the ESR spectrum of monoradical **1** (X = NO_3) is given in dashed line) and DNA modified with complex **2c** at 77 K (*2*) and ~298 K (*3*).

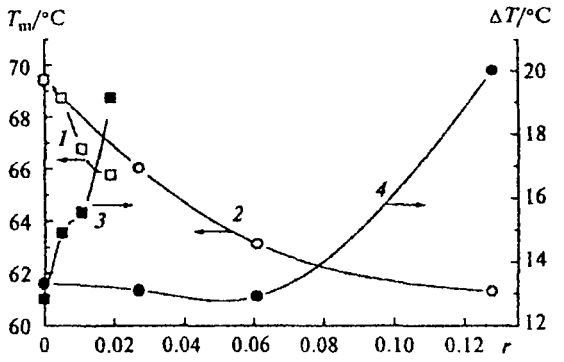


Fig. 3. Dependences of melting point T_m (*1*, *2*) and the width of melting range ΔT (*3*, *4*) on the degree of DNA modification (r) with complex **2c** (*1*, *3*) and cisplatin (*2*, *4*).

nitrate complex **1** (X = NO_3) platinates DNA under similar conditions even more actively (r = 0.16) than cisplatin does.[3] Platination occurs as the result of the replacement of the ligands X mainly by N(7) atoms of guanine and adenine, which are located in the large groove of the B form of DNA.[11] The width of this groove (2.3 nm) suggests that two bulky APO ligands in complex **2c** can exert steric hindrances to platination, whereas one DAPO ligand of complexes **1** does not hinder platination of DNA.[3] Quantum-chemical calculations of substituted 1,2-diaminocyclohexanes as examples demonstrated that the steric arrangement of the substituents in platinum diaminoligands also substantially influences the efficiency of platination of DNA.[12]

Because of strong exchange and dipole interactions between unpaired electrons, the ESR spectra of DNA modified with biradical **2c** are observed as broad lines without pronounced features at both 77 K and room temperature (see Fig. 2, curves *2* and *3*). The intensity of ESR lines of modified DNA is low because of their considerable width and low degree of DNA modification.

The melting (denaturation) point T_m and melting range ΔT of DNA in solution characterize the stability of the duplex and the scatter in strength of hydrogen bonds that form the duplex, respectively. These characteristics are quite sensitive to disturbance of the secondary structure of DNA due to the formation of platinum adducts. The profiles of dependences of T_m and ΔT on the degree of platination r for **2c** and cisplatin are shown in Fig. 3.

Unexpectedly, it turned out that **2c** and cisplatin decrease T_m in a similar way but influence ΔT in a very different manner. While cisplatin decreases T_m and almost does not influence ΔT up to $r \approx 0.07$, the ΔT value for **2c** increases strongly with increase in r. This attests to a sharp increase in heterogeneity of the DNA structure upon formation of $Pt^{II}(APO)_2$—DNA adducts. Strong disordering of the DNA structure with adducts **2c** is also evidenced by the degree of hyperchromicity of the duplex during melting. At r = 0.02—0.03, which

corresponds to two to three adducts per every 100 nucleotides, the decrease in the degree of hyperchromicity of DNA modified with cisplatin and **2c** is equal to 3±1 and 13±2%, respectively.

Data on overall toxicity and antitumor activity of the complexes are given in Table 1. Data published[13] and obtained by us[3] indicate that for complexes in which the bulk of aminoligands does not exceed certain value a correlation is observed: the higher the rate of hydrolysis of the leaving X ligands the higher the toxicity of the complexes. Data from Table 1 show that complexes **2a—d** do not follow this tendency because the rate of hydrolysis usually increases in the series halide < oxalate < nitrate complex,[3] and in our case, toxicity increases in the series chloride < nitrate < oxalate < iodide complex. Two bulky APO ligands strongly hinder platination of DNA and other nucleophilic centers, which is likely the reason for the significant decrease in the toxicity of **2a—d** compared to complexes **1** with the same X ligands.[2] Among complexes **2**, the most lipophilic **2b** has maximum toxicity and, when taken in nontoxic doses, does not inhibit the development of leukemia P 388. The moderate antitumor effects of other complexes are probably due to their poor platinating activity relative to DNA. In addition, data[14] on reparation of platinated DNA suggest greater ease of reparation for adducts that cause greater disturbance of the DNA structure, which is characteristic of biradical complexes **2**.

Thus, it is evident that the bulk of transport aminoligands in platinum complexes can substantially change their platinating activity with respect to DNA as well as the properties of the adducts formed. Higher activity in platination of DNA and lesser destabilization of duplex in the formation of adducts should be expected for complexes with less bulky ligands than those in **2**. According to preliminary data, such properties are exhibited by mixed-ligand complexes of the general formula *cis*-$Pt^{II}(APO)(NH_3)X_2$ that we have synthesized recently. The results of their study will be reported elsewhere.

This work was financially supported by the International Scientific and Technical Center (Project No. 123-94).

References

1. M. J. Bloemink and J. Reedijk, in *Metal Ions in Biological Systems*, Eds. H. Sigel and A. Sigel, Marcel Dekker, New York, 1996, **32**, p. 641.
2. V. D. Sen', V. A. Golubev, L. M. Volkova, and N. P. Konovalova, *J. Inorg. Biochem.*, 1996, **64**, 69.
3. A. V. Shugalii, A. V. Kulikov, M. V. Lichina, V. A. Golubev, and V. D. Sen', *J. Inorg. Biochem.*, **69**, 67.
4. A. Mathew, B. Bergquist, and J. Zimbrick, *J. Chem. Soc., Chem. Commun.*, 1979, 222.
5. Y.-Y. H. Chao, A. Holtzer, and S. H. Mastin, *J. Am. Chem. Soc.*, 1977, **99**, 8024.
6. E. G. Rozantsev, *Svobodnye iminoksil'nye radikaly* [*Free Iminoxyl Radicals*], Khimiya, Moscow, 1970, 200 (in Russian).
7. B. Zimm and D. Crothers, *Proc. Natl. Acad. Sci. USA*, 1962, **48**, 905.
8. F. Cotton and G. Wilkinson, *Advanced Inorganic Chemistry*, Wiley, New York, 1966, **1**.
9. K. Nakamoto, *Infrared and Raman Spectra of Inorganic and Coordination Compounds*, Wiley, New York, 1986.
10. V. N. Parmon, A. I. Kokorin, and G. M. Zhidomirov, *Stabil'nye biradikaly* [*Stable Biradicals*], Nauka, Moscow, 1980, 240 (in Russian).
11. S. J. Lippard, *Pure Appl. Chem.*, 1987, **59**, 371.
12. K. J. Miller, S. L. McCarthy, and M. Krauss, *J. Med. Chem.*, 1990, **33**, 1043.
13. P. Schwartz, S. J. Meischen, G. R. Gale, L. M. Atkins, A. B. Smith, and E. M. Walker, *Cancer Treat. Rep.*, 1977, **61**, 1519.
14. M. Takahara, A. C. Rosenzweig, C. A. Frederick, and S. J. Lippard, *Nature*, 1995, **377**, 649.

Received December 26, 1997;
in revised form February 6, 1998