Synthesis, Crystal Structure and Biological Characterization of a Novel Palladium(II) Complex with a Coumarin-Derived Ligand

Elzbieta Budzisz,*[a] Bernhard K. Keppler,[b] Gerald Giester,[c] Magdalena Wozniczka,[d] Aleksander Kufelnicki,[d] and Barbara Nawrot[e]

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Methyl 2-methyl-4-oxo-4*H*-chromene-3-carboxylate (**1**) was treated with 25% aqueous ammonia to give 3-(1-aminoethylidene)-2*H*-chromene-2,4(3*H*)-dione (**2**). Compound **2** was used as a ligand for the formation of a palladium(II) complex. The structures of the ligand and its palladium complex **3** were determined by IR and ^{1}H NMR spectroscopy, FAB mass spectrometry and elemental analysis. The single-crystal X-ray structure of **3** was also solved. Ligand **2** in 20% dioxane solution shows two protonation constants — $\log \beta_{11} = 4.28 \pm 0.01$ and $\log \beta_{12} = 7.66 \pm 0.03$. In complex **3** two ligand molecules chelate to the Pd ion through their N and O donors, giving a four-coordinate Pd^{II} center with a *cis*-N_2O_2 donor set. The coordination geometry at Pd^{II} is square-planar with typical values for the distances from the Pd atom to the coordinated atoms. The cytotoxicity of compound **3** was determined on three cancer cell lines. IC_{50} values of 9.7, 7.8 and 7.8 nM were found for A546, HeLa and K562 cells, respectively, which is notably lower than carboplatin, which shows corresponding IC_{50} values of 105.98, 73.49 and 60.92 μM. Double-stranded DNA treated with **3** shows a significant increase in the Δε value in the CD spectra, as well as a lower electrophoretic mobility than parent DNA.

Introduction

Since the discovery by Rosenberg[1] that cisplatin exhibits antitumour activity, extensive studies on platinum complexes with various ligands as potential antitumor agents have been performed. It has been shown that cisplatin [*cis*-diamminedichloroplatinum(II)] is active against several human malignant diseases, such as neck and head, ovarian, testicular, lung, and bladder cancers.[2] It is generally accepted that cisplatin exerts its therapeutic activity by binding directly to DNA, deactivating it as a template for the transcription process and inhibiting in that way the protein biosynthesis.[3] However, cisplatin has several disadvantages, among which are its severe nephrotoxicity, neurotoxicity and emetogenesis.[4] The toxic side-effects of cisplatin therefore limit the doses that can be applied to patients.[5] The clinical application of the second-generation drug diammine[1,1-cyclobutanedicarboxylato(2−)-*O*,*O*′]platinum(II) (carboplatin; Figure 1) resulted in significant decrease of the toxic side-effects. These differences were attributed to the greater pharmokinetic stability of carboplatin due to the presence of the bidentate bis(carboxylato) ligand.[6] Studies on the interaction of carboplatin with DNA indicate that the mechanism involves carboplatin ring-opening and subsequent platinum binding to DNA nucleobases.[7]

Taking into account the structural analogy between Pt^{II} and Pd^{II} complexes, a variety of studies on Pd^{II} complexes have been performed, including their cytotoxicity[8] and antitumor activity.[9−11] Pd complexes with β-carboline alkaloids,[12−14] pyrazoles[15] and DMSO[16] have been tested against solid-tumor cell lines, and in some cases exhibited remarkable activity. It has been suggested that their biological activity depends on the nature of the ligand, the type of the counterion used and the configuration of the complex.

Coumarin and its derivatives are biologically important compounds with spasmolytic, antiarrhythmic, cardiotonic,

[a] Faculty of Pharmacy, Medical University of Lodz,
Muszyńskiego 1, 90-151 Lodz, Poland
Fax: + 48-42-678-83-98
E-mail: elora@ich.pharm.am.lodz.pl
[b] Institute of Inorganic Chemistry of the University of Vienna,
Währingerstr. 42, 1090 Vienna, Austria
E-mail: keppler@ap.univie.ac.at
[c] Institute of Mineralogy and Crystallography of the University of Vienna,
Althanstr. 14, 1090 Vienna, Austria
E-mail: gerald.giester@univie.ac.at
[d] Laboratory of Physical and Biocoordination Chemistry, Faculty of Pharmacy, Medical University of Lodz,
Muszyńskiego 1, 90-151 Lodz, Poland
Fax: + 48-42-678-83-98
E-mail: akuf@ich.pharm.am.lodz.pl
[e] Department of Bioorganic Chemistry, Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences,
Sienkiewicza 112, 90-363 Lodz, Poland
Fax: + 48-42-681-54-83
E-mail: bnawrot@bio.cbmm.lodz.pl

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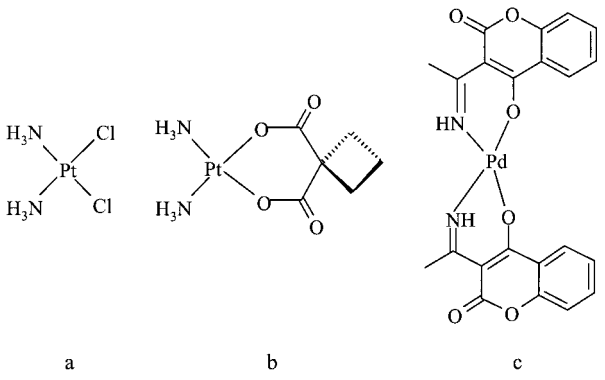


Figure 1. The structures of cisplatin (a), carboplatin (b) and complex **3** (Pd^{II} complex of ligand **2**) (c)

photodynamic[17] and antitumor properties.[18,19] Metal complexes of coumarin reveal anticoagulant action[20,21] and possess antitumor activity.[22,23] In particular, complexes of coumarin with cerium(III), zirconium(IV), copper(II), zinc(II), bismuth(III) and cadmium(II) have shown promising cytotoxic activity in vitro.[24,25]

Here, we describe the synthesis, structure and properties of a novel coumarin ligand **2** and its palladium(II) complex **3** (Figure 1). Spectral data and protonation constants are given for the ligand **2**. The structure of complex **3** is confirmed by spectral and X-ray analysis. Its biological properties are evaluated, including alkylating activity, cytotoxicity against selected cell lines as well as its interaction with double-stranded DNA monitored by circular dichroism (CD) spectroscopy and agarose-gel electrophoresis.

Results and Discussion

Synthesis and Structure of Ligand 2

The reaction of methyl 2-methyl-4-oxo-4*H*-chromene-3-carboxylate[26] (**1**) with an equimolar amount of 25% aqueous ammonia in methanol afforded product **2** in 94% yield (Scheme 1). This compound was previously synthesized by Somogyi and co-workers[27] in a multistep reaction with a total yield of 23% starting from 3-acetyl-4-hydroxycoumarin. Our approach is based upon a one-step, high-yield transformation of chromone **1** into 3-substituted coumarin **2** by the reaction of **1** with ammonia or an alkylamine.[28]

The structure of compound **2** was confirmed by spectroscopic methods (^1H and ^{13}C NMR, IR, EI-MS) as well as by elemental analysis. The first report of this compound by Somogyi et al. suggested that compound **2** exists in two tautomeric forms **2** and **2a** (Scheme 1), with proton magnetic resonances at δ = 10.01 (NH) and 12.04 (OH) ppm, respectively. These data were confirmed by our investigations, as two signals at δ = 9.99 and 12.32 ppm are observed in the ^1H NMR spectrum of **2** taken in DMSO. These signals disappear after proton exchange with D_2O, which directly confirms the presence of both tautomeric forms in DMSO solution. However, *N*-alkyl-substituted derivatives of **2** exist exclusively in the keto form in the solid state and in solution.[29–31] Thus, the presence of a β-amino group in the α,β-unsaturated carbonyl system ensures the accessibility of both terminal centers for metal-ion complexation.

Determination of the Protonation Constant of Ligand 2

Potentiometric experiments within the pH range 2.5–5.5 showed that the ligand behaves as a diprotonated molecule with one hydroxy (OH) and one imino (NH) proton. The measured protonation constants $\log \beta_{11} = 4.28 \pm 0.01$ and $\log \beta_{12} = 7.66 \pm 0.03$ are in reasonable agreement with those obtained from theoretical calculations[32] ($\log \beta_{11} = 4.50 \pm 0.20$ and $\log \beta_{12} = 7.71 \pm 0.20$, respectively). The statistical parameters $\sigma = 3.52$ and $\chi^2 = 15.64$ only slightly exceed the critical values of 3.00 and 12.60 at a confidence level of 0.95 as regarding the comprehensive file consisting of four titration data, but they are satisfactory in the particular titrations.

The two protonation constants indicate the presence of two potential donors, the 4-O and 3-N atoms. According to our results, the stepwise protonation constants are 4.28 and 3.38. An exemplary speciation diagram is shown in Figure 2.

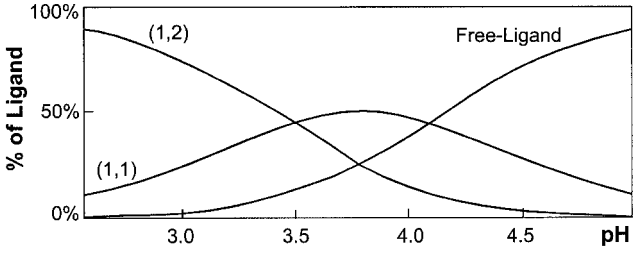
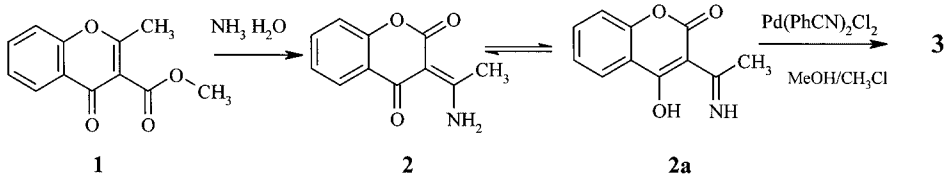


Figure 2. Species distribution of L = 4-hydroxy-3-(1-iminoethyl)-2*H*-chromen-2-one in 20% dioxane; (1,1) and (1,2) denote the mono- and diprotonated forms of L^{2-} (free ligand); $C_L = 6.21 \times 10^{-3}$ mol·dm^{-3}; $\sigma = 1.75$; $\chi^2 = 9.58$



Scheme 1. Synthesis of the ligand **2** and its Pd complex **3**

Synthesis and Structure of Complex 3

The palladium(II) complex of ligand **2** was synthesized by mixing **2** and bis(benzonitrile)dichloropalladium(II) in a 1:2 molar ratio in chloroform/methanol (1:2.5) solution at room temperature for 24 h (Scheme 1). The resulting solid shows an FAB-MS signal at $m/z = 510$, characteristic for PdL_2. This complex composition was confirmed by elemental analysis. In the ^1H NMR spectrum of the complex, one of the exchangeable protons, attributed to the enol OH group (at $\delta = 12.32$ ppm) of **2a**, is not present. The IR spectrum excludes the presence of chloride groups (no signals at 350 cm^{-1}). Instead, two bands were found at 508 and 437 cm^{-1}, which could be assigned to the M−O and M−N vibrations.[33] Moreover, the band assigned to the NH vibrations in **2** (3260 cm^{-1}) is shifted to higher energy (3310 cm^{-1}), indicating that the nitrogen atom is involved in the coordination (Table 1). At this stage we could not assign the complex geometry. It is known that palladium(II) complexes adopt predominantly *trans* conformations,[34] and only a few examples of *cis* conformers are known.[35]

Table 1. IR frequencies ($\tilde{\nu}$ [cm^{-1}]) for the ligand and its palladium complex

	NH/OH	C=O	C=N	M−N	M−O
2	3261	1708 1665	1609	–	–
3	3310 3265	1702 1670	1606	437	508

Our attempts to obtain a platinum(II) complex of ligand **2** failed, despite trying various solvent systems (aqueous methanol, aqueous acetone, aqueous dioxane, chloroform/methanol), various Pt^{II} substrates {K_2PtCl_4 or *cis*-$[PtCl_2(PhCN)_2]$} and various temperature conditions (room temperature, 60 °C). A black powder, identified as platinum, was the only solid product of this reaction. In contrast, a phosphorus analogue of coumarin (2-ethanimidoyl-2-methoxy-2*H*-1,2-benzoxaphosphinin-4-ol 2-oxide) forms complexes with Pt^{II} and Pd^{II} with a structure analogous to **3**.[36] We cannot offer any explanation why the Pt^{II} complex of ligand **2** is not formed or is too unstable to be isolated.

X-ray Structure of 3

The X-ray diffraction study of **3** (Figure 3) shows that in the PdL_2 molecule two L^- ligands chelate to the Pd ion through their N and O donors, giving a four-coordinate Pd^{II} center with a *cis*-N_2O_2 donor set. The coordination geometry at Pd^{II} is square-planar, with standard values for the distances from the metal atom to the coordinated atoms [Pd−N1: 1.965(6); Pd−N2: 1.951(6); Pd−O1: 1.972(5); Pd−O4: 1.990(5) Å]. The Pd^{II} ion lies in the least-squares plane of the coordinated atoms, deviating by ±0.023 Å. While the C2−N1 and C13−N2 bonds in the six-membered chelate rings possess the character of a localized double bond that deviates only marginally from the ideal value of 1.28 Å,[37] a substantial lengthening of the C11−O1 and C22−O4 bonds is observed when compared to the localized double bonds C4−O2 and C15−O5 (Table 2). The distribution of the bond lengths over the fragments C2−C3−C11 and C13−C14−C22 indicates significant delocalization of the electron density (Table 2).

Table 2. Bond lengths [Å] and angles [°] for compound **3**

Pd(1)−N(2)	1.951(6)	C(11)−O(1)−Pd(1)	128.1(4)
Pd(1)−N(1)	1.965(6)	C(5)−O(3)−C(4)	123.1(6)
Pd(1)−O(1)	1.972(5)	C(22)−O(4)−Pd(1)	127.1(4)
Pd(1)−O(4)	1.990(5)	C(16)−O(6)−C(15)	122.8(6)
N(2)−Pd(1)−N(1)	92.8(3)	C(2)−N(1)−Pd(1)	131.5(5)
N(2)−Pd(1)−O(1)	177.0(2)	C(2)−N(1)−H(1)	114.2
N(1)−Pd(1)−O(1)	89.6(2)	Pd(1)−N(1)−H(1)	114.2
N(2)−Pd(1)−O(4)	89.6(2)	C(13)−N(2)−Pd(1)	131.5(5)
N(1)−Pd(1)−O(4)	177.5(2)	C(13)−N(2)−H(2)	114.3
O(1)−Pd(1)−O(4)	88.1(2)	Pd(1)−N(2)−H(2)	114.3

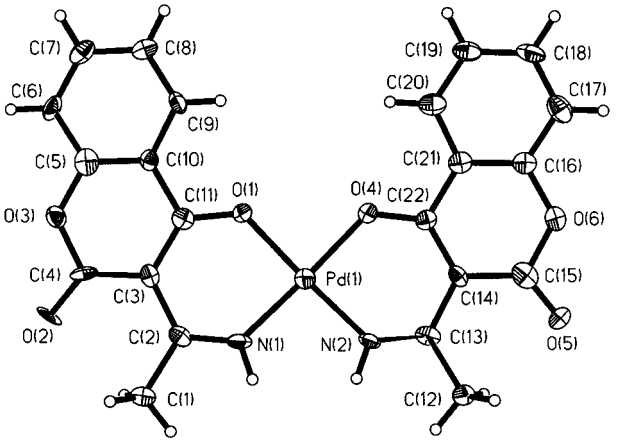


Figure 3. ORTEP drawing of the molecule of **3** with thermal ellipsoids depicted at 50% probability

The molecular structure of **3** shows stacked pairs with an interplanar "face-to-back" separation of 3.28 Å; the Pd1···Pd1′ distance is 3.775 Å. The shortest contact observed is Pd1···O4′ at 3.566 Å and the angle O4′···Pd1′···Pd1 is 80.1°. These pairs, which are slightly slipped with respect to each other, form columns with separations between pairs of 3.37 Å and a Pd1···Pd1′′ separation of 6.736 Å.

Biological Characterization of 3

Alkylating Properties

Cisplatin and carboplatin are nonclassical alkylating agents. These drugs interact with cellular DNA according to the same mechanism[38,39] — alkylation of nucleobases of both DNA strands. An effective drug concentration for carboplatin is 4−7 times higher than for cisplatin, and the former is also less toxic.[40] The alkylating activity of complex **3** was determined according to the Preussmann test.[41] The level of alkylation of 4-(4′-nitrobenzyl)pyridine (NBP) was quantified spectrophotometrically at 560 nm. The molar

absorbance (A) for complex **3** is 0.3584, while for ligand **2** it is significantly lower (Table 3). Interestingly, cisplatin in this test has an A value of 0.300[42] and carboplatin alkylates NBP with an A value of 0.2312. According to the Preussmann scale (described in a footnote to Table 3), complex **3** and both cisplatin and carboplatin exhibit moderate alkylating activity in this test.

Table 3. Alkylating activity of compound **2** and its Pd complex **3** determined by the Preussmann test

Compound	Absorbance (A)[a] λ_{max} 560 nm	Alkylation activity[b]
2	0.0508	+
3	0.3584	++
Cisplatin[c]	0.3000	++
Carboplatin	0.2312	++

[a] Means of three determinations. [b] According to Preussmann: (+) A = 0.05−0.1, (++) A = 0.1−0.5. [c] According to Zyner.[42]

Cytotoxicity

The cytotoxicity of **2** and **3** was determined in a concentration range from 10^{-10} to 10^{-3} M using three human cell lines — A549, HeLa and K562 — with carboplatin as reference. The activity is expressed as the concentration required for reducing the cell survival fraction to 50% after 72 h exposure of the cells to the compounds (IC_{50}; Table 4). The responses of A549, HeLa and K562 cells to continuous exposure to **2** and **3** at various concentrations for 24, 48 and 72 h are presented in Figure 4. From these figures, a time-dependent response can be observed for complex **3** in all three cell lines. Interestingly, compound **3** exhibits very high cytotoxicity against A549, HeLa and K562 cells, with IC_{50} values of 0.0097, 0.0078 and 0.0078 μM, respectively, which are superior to those of the reference carboplatin (105.98, 73.49 and 60.92 μM). The ligand was not toxic for the test cell lines even after 72 h exposure.

Table 4. IC_{50} values of compounds **2**, **3** and carboplatin after 72 h

Compound	A549 IC_{50} [μM][a]	HeLa IC_{50} [μM][a]	K562 IC_{50} [μM][a]
2	> 1000[b]	> 1000[b]	> 1000[b]
3	0.0097 ± 0.0017	0.0078 ± 0.0003	0.0078 ± 0.0007
Carboplatin	105.98 ± 6.79	73.49 ± 6.03	60.92 ± 13.64

[a] Mean values of IC_{50} (in μM) ± standard deviation from four experiments are presented. [b] At this concentration of compound **2** reduction in cell growth was less than 50%.

Interaction of 3 with Double-Stranded DNA

CD Analysis

The influence of complex **3** on the geometry of double-stranded DNA was investigated by CD spectroscopy. The CD spectra of the control samples (DNA in PBS buffer and DNA in PBS buffer/10% DMSO) were compared with the spectra of DNA incubated at room temperature for 18 h with increasing amounts of complex **3** (Figure 5). For DNA in PBS buffer the maximum intensity of the positive band at 281 nm was found at $\Delta\varepsilon$ = 6.2; for DNA in PBS/10% DMSO solution this value was 7.0. The spectra of the control samples do not differ significantly, indicating that at this concentration (10%) DMSO has no influence on the DNA structure. However, changes are observed in the spectra of DNA incubated with complex **3**. While the λ_{max} value of the positive band is not shifted, the $\Delta\varepsilon$ value is lowered

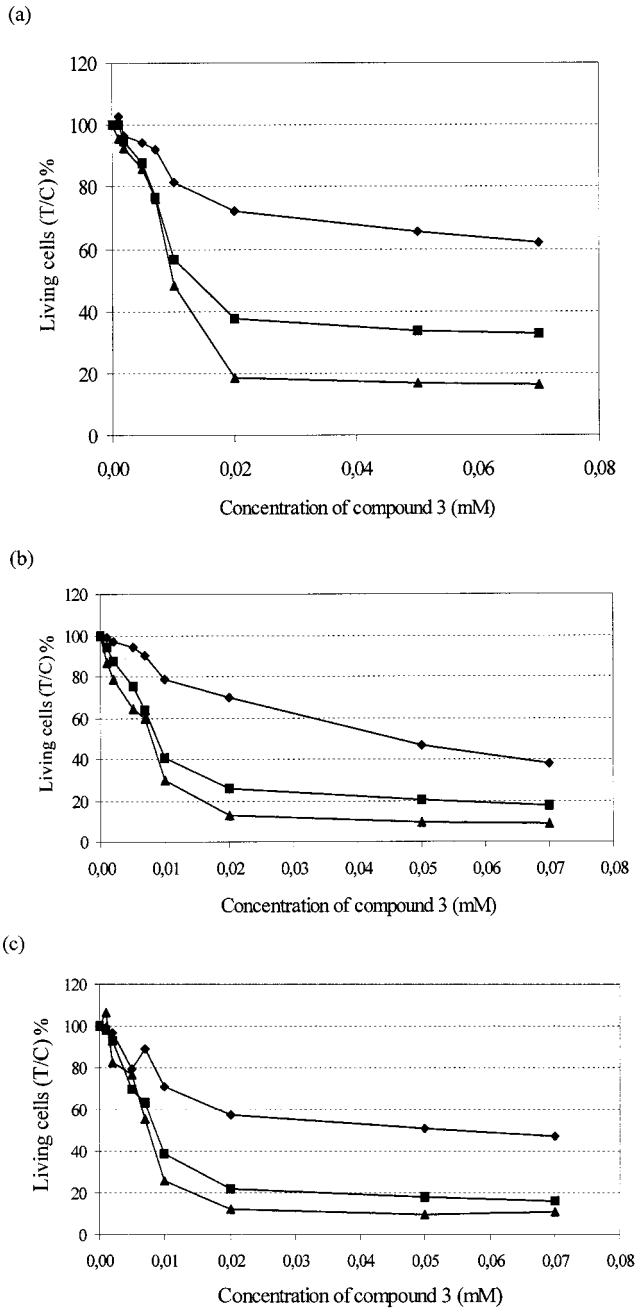


Figure 4. Cytotoxic effect of complex **3** on (a) A549, (b) HeLa, and (c) K562 cell lines after 24 (diamonds), 48 (squares) and 72 h (triangles) measured by MTT assay

by 0.8 units for DNA interacting with complex **3** at r_i = 1.3. At r_i = 2.6 and r_i = 3.9 (r_i is the molar ratio of **3** to nucleotides) the $\Delta\varepsilon$ values increase to 19 and 34 units, respectively, with only a 2 nm shift of the λ_{max} value to long wavelength, which suggests that structural changes are produced on DNA by complex **3**. These changes may originate from several sources, such as intercalation of the palladium compound into double-stranded DNA[43] or interhelical alkylation of nucleobases.[44] Such a significant increase of band intensity accompanied by the shift of the long-wavelength crossover by about 3 nm is characteristic for denatured DNA structures.[45] It is also known that dehydration of DNA, which induces $B \rightarrow A$ conformational changes, results in an increase of the band intensity in the region between 250 and 300 nm.[45] However, dehydration of DNA is not likely here since the concentration of complex **3** (r_i = 3.9) and DMSO (10%) is much lower than the conditions required to induce such conformational changes (> 60% ethanol).

DMSO (%)	–	–	10	20	30	10	20	30
Preincubation	–	+	+	+	+	+	+	+
Complex **3** [μg]	–	–	–	–	–	0.1	0.2	0.3
r_i						0.2	0.4	0.6
Lane M	1	2	3	4	5	6	7	8

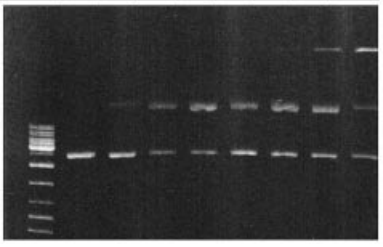


Figure 5. Interaction of **3** with double-stranded DNA monitored by 3% agarose gel electrophoresis; plasmid DNA (pUC, 2749 bp) was incubated with increasing amounts of complex **3**/DMSO in 3 × diluted PBS buffer containing 10 mM $MgCl_2$; M: mass marker; lanes 1 and 2: DNA in PBS buffer without and with 18 h, incubation; lanes 3–5: DNA in PBS buffer with increasing amounts of DMSO (as described above); lanes 6–8: DNA in PBS with increasing amounts of complex **3** and DMSO (data given above figure)

Agarose-Gel Electrophoresis

Plasmid DNA samples dissolved in triply diluted PBS/10 mM $MgCl_2$ buffer were incubated for 18 h at room temperature with increasing amounts of complex **3** (DMSO solution). As controls, plasmid DNA in PBS buffer and in PBS/DMSO buffer (containing 10, 20 or 30% of DMSO) were used. Samples were analyzed by agarose-gel electrophoresis (Figure 6). At first we checked the impact of incubation on DNA structure. After 18 h of incubation, we observed more of the superhelical form (90%) and only a very small amount of the circular form of DNA (10%) (lane 2 versus lane 1). In the plasmid samples incubated with increasing amounts of DMSO, the ratio of both forms of DNA was almost identical (lanes 3–5). However, when DNA was treated with increasing amounts of complex **3**, we observed a new band of very low electrophoretic mobility, tentatively attributed to a high-molecular-weight DNA/**3** adduct which contains 13.2% and 48.6% of the total DNA (lanes 7 and 8, respectively). The observed lowered mobility might result from intercalation of the planar compound **3** into the double-stranded plasmid DNA.

Conclusions

Coumarin-derived palladium(II) complex **3** is an interesting analogue of carboplatin. Its very high cytotoxicity toward three cancer-cell lines, with IC_{50} values in the nanomolar range (four orders of magnitude lower than that for the reference carboplatin), speaks for the need for further biological evaluations. At the present stage we can conclude that **3**, similarly to cisplatin and carboplatin, shows moderate alkylating activity toward aromatic amine (NBP), a compound that mimics the nucleobases in DNA. Complex

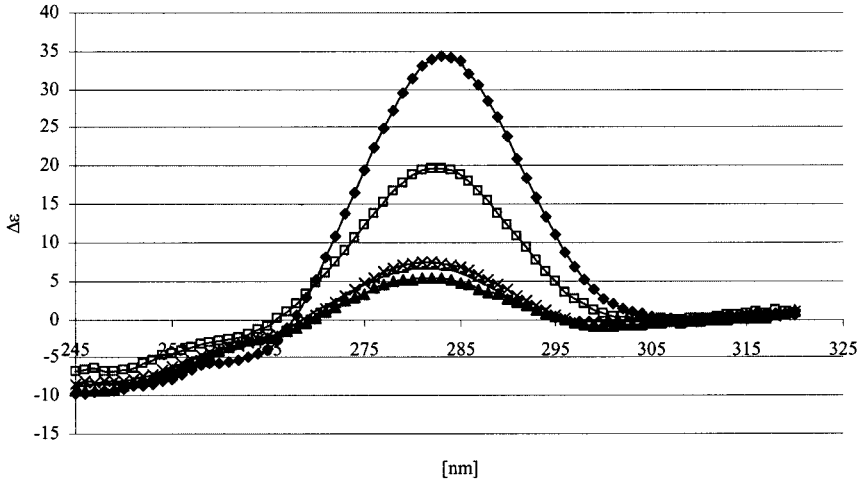


Figure 6. CD spectra of control double-stranded DNA (20 bp duplex of sequence given in Exp. Sect.) in PBS buffer (-) and in PBS/10% DMSO buffer (×), and adducts of DNA with increasing amount of complex **3** r_i = 1.3 (triangles), r_i = 2.6 (empty squares) and r_i = 3.9 (diamonds)

3 probably also binds to double-stranded DNA, enforcing in that way structural changes of short duplex DNA and reducing the electrophoteric mobility of plasmid DNA.

Experimental Section

General: The melting points were determined using an Electrothermal 1A9100 apparatus and are uncorrected. The IR spectra were recorded with a Pye-Unicam 200G Spectrophotometer in KBr and CsI. The ^{1}H NMR spectra were registered at 300 MHz with a Varian Mercury spectrometer. The MS data were obtained with a LKB 2091 mass spectrometer (70 eV ionisation energy). The MS-FAB data were determined with a Finnigan Matt 95 mass spectrometer (NBA, Cs^+ gun operating at 13 keV). For the new compounds satisfactory elemental analyses (±0.3% of the calculated values) were obtained using a Perkin−Elmer PE 2400 CHNS analyser. Methyl 2-methyl-4-oxo-4*H*-chromene-3-carboxylate (**1**) was prepared according to a published method.[26]

Synthesis of 3-(1-Aminoethylidene)-2*H*-chromene-2,4(3*H*)-dione (2): A 25% aqueous ammonia solution (0.31 mL, 2 mmol) in methanol (0.5 mL) was added at room temperature to a solution of methyl 2-methyl-4-oxo-4*H*-chromene-3-carboxylate (**1**; 0.44 g, 2 mmol) in methanol (5 mL). After several minutes, the solid crude product precipitated. The product was filtered off, dried, and crystallized from acetone (5 mL). Pure compound **2** was obtained as a white solid (382 mg, 94.0%); m.p. 234.5−234.9 °C. ^{1}H NMR ([D$_6$]DMSO): δ = 2.58 (s, 3 H, CH$_3$), 7.23−7.94 (m, 4 H, aromat. H), 9.99 (s, 1 H, NH), 12.32 (s, 1 H, OH) ppm. ^{13}C NMR ([D$_6$]DMSO): δ = 24.57 (C−CH$_3$), 95.57, 116.15, 120.24, 123.43, 125.59, 133.83, 152.95, 161.79, 177.06, 179.36 (C=O) ppm. MS: *m*/*z* (%) = 204 (100) [M$^+$], 124 (1.75). $C_{11}H_9NO_3$ (203.19): calcd. C 65.02, H 4.46, N 6.89; found C 65.05, H 4.45, N 7.07.

Palladium Complex 3: A methanolic solution of **2** (0.5 mmol, 101.6 mg in 5 mL) was added to a chloroform/methanol (1:1, v/v; 5 mL) solution of [PdCl$_2$(C$_6$H$_5$CN)$_2$] (95.9 mg, 0.25 mmol). The mixture was stirred at room temperature. After 2 h, the resulting light yellow crystals were filtered off, washed with diethyl ether, and dried in vacuo over anhydrous silica gel overnight. Yield 126.3 mg (43.3%); m.p. 330 °C (dec.). ^{1}H NMR ([D$_6$]DMSO): δ = 2.64 (s, 6 H, CH$_3$), 7.29−8.16 (m, 8 H, aromat. H), 9.43 (s, 2 H, NH) ppm. MS-FAB: *m*/*z* = 511.2. $C_{22}H_{16}N_2O_6Pd$ (510.72): calcd. C 51.73, H 3.16, N 5.48; found C 51.69, H 3.16, N 5.29.

Potentiometric Studies of the Ligand: The protonation constants of the ligand were determined by pH-metric titration of 4-mL samples at a temperature of 25 ± 0.1 °C. The total concentration of the ligand in each sample ranged from 6.0×10^{-3} to 2.0×10^{-2} M. Owing to the very low solubility in pure water a mixed 20% (v/v) 1,4-dioxane/water solvent was used. The titrations were carried out with a carbonate-free NaOH solution of known concentration (0.5 mol/L). The value of pK_w = 13.77 was obtained from our acid/base calibrations in the same solvent. The pH was measured with a Molspin Ltd. (Newcastle upon Tyne, England) automatic titration set and combined OSH 10-10 electrode (Metron, Poland). The total volume of the Hamilton microsyringe in the autoburette was 250 μL; the volume increments amounted to 0.0025 mL. The experiments were performed using MOLSPIN.EXE software. The electrode was calibrated in the −log [H$^+$] scale by titration of 0.005 M HNO$_3$ (in 20% dioxane) with 0.5 M NaOH at 25 °C. Then overall protonation constants $\beta_{lh} = [L_lH_h]/[L]^l[H]^h$ were calculated with the SUPERQUAD computer program.[46]

X-ray Crystallographic Structure Determination:[47] X-ray diffraction measurements were performed with a Nonius Kappa CCD diffractometer at 120 K. A colorless single crystal was positioned at 30 mm from the detector and 137 frames were measured, each for 200 s over a 2° scan. The data were processed using Denzo-SMN software.[48] Crystal data, data collection parameters, and structure refinement details for **3** are given in Table 5. The structure was solved by direct methods and refined by full-matrix least-squares techniques. Non-hydrogen atoms were refined with anisotropic displacement parameters. The H atoms were placed in calculated positions and allowed to ride. Computer programs: structure solution SHELXS-97;[49] refinement SHELXL-97;[50] molecular diagrams ORTEP;[51] computer: Pentium II; scattering factors were taken from the literature.[52]

Table 5. Crystal data and details of data collection for **3**

	3
Empirical formula	$C_{22}H_{16}N_2O_6Pd$
Formula mass	510.77
Space group	$P2_1/n$
a [Å]	9.384(2)
b [Å]	11.241(2)
c [Å]	18.306(4)
β [°]	103.38(3)
V [Å^3]	1878.6(7)
Z	4
λ [Å]	0.71073
$\rho_{calcd.}$ [g cm^{-3}]	1.806
Crystal size [mm]	0.025 × 0.025 × 0.3
T [K]	120
μ [cm^{-1}]	10.34
*R*1[a]	0.0376
*wR*2[b]	0.0942
GOF[c]	1.002

[a] $R1 = \Sigma||F_o| - |F_c||/\Sigma|F_o|$. [b] $wR2 = \{\Sigma[w(F_o^2 - F_c^2)^2]/\Sigma[w(F_o^2)^2]\}^{1/2}$. [c] GOF = $\{\Sigma[w(F_o^2 - F_c^2)^2]/(n - p)\}^{1/2}$, where *n* is the number of reflections and *p* is the total number of parameters refined.

Cell Lines and Culture Conditions: Three human cell lines were used for cytotoxicity experiments: A549 (lung carcinoma, human), HeLa (cervix epithelial carcinoma, human), K562 (chronic myelogenous leukemia, human). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, streptomycin (100 μg/mL) and penicillin (100 U/mL) at 37 °C in 5% CO_2/95% air.

Drug Cytotoxicity: Exponentially growing cells were seeded at $5-7 \times 10^3$ in 200 μL of the complete culture medium per well of a 96-well plate (Nunc). After 24 h, the cells were exposed to compounds **2**, **3** and carboplatin (used as a reference) at 37 °C in 5% CO_2/95% air for 24 h, 48 h and 72 h. Cytotoxicity was estimated for a concentration range from 10^{-10} to 10^{-3} M. The cell number relative to the control was then determined by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma][53] method. The values of IC_{50} (concentration of compounds required to reduce the cell survival fraction to 50% of the control) were calculated from dose-response curves and used as a measure of cellular sensitivity to a given treatment. Each experiment was performed in quadruplicate and evaluation was based on means.

Determination of Alkylating Properties: The test compound (0.005 mmol) was dissolved in 2-methoxyethanol (1 mL) and a solution of 4-(4′-nitrobenzyl)pyridine (NBP) in 2-methoxyethanol

(5% solution, 1 mL) was added. The sample was heated at 100 ± 0.5 °C for 1 h and then quickly cooled to 20 °C. 2-Methoxyethanol (2.5 mL) and piperidine (0.5 mL) were added to the sample to give a total volume of 5 mL. The final concentration of the test compound was 1×10^{-3} M. After 90 s, the absorbance was measured at λ_{560} nm in a glass cell (1 cm). 2-Methoxyethanol was used as a reference solvent.

CD Spectra of Complex 3 with Double-Stranded DNA: CD measurements of complex **3** with double-stranded DNA were performed with a Jobin Yvon CD6 (France) dichrograph. The double-stranded DNA was obtained by annealing of 5′-TCTTCAAGAATTCAGGACTA-3′ and 5′-TAGTCCTGAATTCTTGAAGA-3′. Samples of double-stranded DNA (4.6×10^{-6} M, 200 μL) were dissolved in PBS buffer (700 μL, 155 mM NaCl, 4.2 mM KH_2PO_4/Na_2HPO_4, pH = 7.2), to which the complex **3** in a solution of DMSO (1.9×10^{-3} M, 25, 50 or 75 μL) was added. DMSO was added to each sample up to 10% of total volume (including solvent of the complex **3**). The molar ratio of compound **3** to nucleotides is expressed as r_i. Each sample of a total volume of 1 mL was incubated at room temperature for 18 h and then scanned twice between 245 and 320 nm at 25 °C. The data are expressed as the mean of Δε (in $°\cdot cm^{-1}\cdot M^{-1}$).

Electrophoretic Mobility of Plasmid DNA in the Presence of Complex 3: Plasmid DNA (pUC19, 2749 bp) (0.2 μg) was incubated at 37 °C for 18 h in a mixture of triply diluted PBS/10 mM $MgCl_2$ buffer and DMSO (10%, 20% or 30%, v/v) that either contained complex **3** (0.1, 0.2 or 0.3 μg) or no complex **3** at all. Electrophoretic analysis was done on 3% agarose gel containing ethidium bromide. Quantification of the bands in the gel was done by densitometry and Image Quant analysis.

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