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Synthesis, Characterization and Stat3 Inhibitory Properties of the Prototypical Platinum(IV) Anticancer Drug, $[PtCl_3(NO_2)(NH_3)_2]$ (CPA-7)

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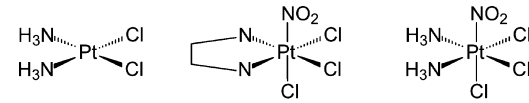
This paper describes a reinvestigation of the literature concerning the synthesis and structural characterization of the platinum(IV)-based anticancer drug known as CPA-7 and believed to be the compound *fac*-$[PtCl_3(NO_2)(NH_3)_2]$. CPA-7 has previously been extensively investigated for its ability to control tumor cell growth by inhibition of Stat3 signaling, but very little information is available concerning its synthesis or spectroscopic properties. A reproducible synthetic route is shown to produce an active material which is characterized by IR and ^{1}H, ^{14}N, ^{15}N, and ^{195}Pt NMR spectroscopy, and single crystal X-ray crystallography. The freshly prepared drug is obtained as a single isomer which may in fact be *fac*- or *mer*-$[PtCl_3(NO_2)(NH_3)_2]$, but recrystallization resulted in a disordered crystal containing approximately equal amounts of the two geometric isomers.

Introduction

Although the prototype platinum-based antitumor drug is *cis*-$[PtCl_2(NH_3)_2]$ (cisplatin),[1] a series of platinum(IV) compounds, such as $PtCl_4$ and the nitro compounds CPA-1 and CPA-7, have also recently been found[2] to be promising inhibitors of the signal transducer and activator of transcription-3 (Stat3), a protein frequently up-regulated in a variety of human cancers.[3a]



The fact that a constitutively active form of Stat3, Stat3C, is able to transform cultured cells further points to an etiologic role for Stat3 in these tumors.[3b] It has also been shown that disrupting hyperactive Stat3 signaling in tumors induces apoptosis with little effect on normal tissues, possibly because tumor cells may have become irreversibly dependent on Stat3 signaling to sustain their growth and survival, while normal ones may be able to use alternate pathways to compensate for Stat3 loss.[3a] As a result, drugs inhibiting Stat3 may be specific for the tumor, with little effect on normal tissues. It was previously shown that CPA-7 does not inhibit dimerization, but it does inhibit binding of Stat3 to DNA[2f] or to the activated EGF-receptor.[3d]

The investigation to be described here began with a request by researchers in the Department of Microbiology and Immunology at Queen's (L.R., A.A.) to researchers in the

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Department of Chemistry at Queen's (S.L.L., M.C.B.) to synthesize research quantities of CPA-7. A subsequent search of the literature for "$[PtCl_3(NO_2)(NH_3)_2]$" turned up a few very old papers and several recent reports on the use of CPA-7 as a Stat3 inhibitor, but very little useful information concerning either synthetic procedures or physical/structural characterization of the compound. We have therefore assessed and modified a rather generic, patented procedure describing the synthesis of a family of Pt(IV) nitro compounds[2c] and have successfully synthesized several batches of a yellow substance, which is found to behave appropriately as a Stat3 inhibitor and which we therefore believe is representative of Stat3 inhibitors used elsewhere.[2g] In an effort to identify the products obtained as containing the compound $[PtCl_3(NO_2)(NH_3)_2]$, with the structure shown above, and to establish criteria by which this compound can be identified, we have also characterized the products obtained by IR spectroscopy, ^{195}Pt, ^{14}N, ^{15}N, and ^{1}H NMR spectroscopy, and single crystal X-ray crystallography. We now report the results of this investigation.

Experimental Section

cis-$[PtCl_2(NH_3)_2]$ (cisplatin), *cis*-$[PtCl_4(NH_3)_2]$, and potassium chloride were purchased from Strem Chemicals, Inc.; nitrogen dioxide (research grade) was purchased from BOC Canada Limited, and $PtCl_4$ was purchased from Aldrich. IR spectra were run on a Perkin-Elmer Spectrum One FT-IR spectrometer. IR analyses were done by dissolving a sample of the compound in the minimum amount of acetone, placing a couple of drops of the solution on a NaCl disk, and then evaporating the acetone as quickly as possible. A pale smear of solid material remained on the disk, and an IR spectrum was run on this solid residue. ^{1}H NMR (DMF-d_7 solutions) spectra were run on a Bruker Avance 500 NMR spectrometer as were ^{195}Pt NMR spectra(107.07 MHz; D_2O solutions); the latter were referenced to external $K_2[PtCl_4]$ in D_2O at $\delta = 0$. ^{14}N NMR spectra (DMSO-d_6 solutions) were run on a Bruker Avance 600 NMR spectrometer (43.36 MHz), with chemical shifts being referenced to nitromethane at $\delta = 380.5$ relative to liquid ammonia at $\delta = 0$.

Synthesis and Characterization of $[PtCl_3(NO_2)(NH_3)_2]$ (CPA-7). In a typical reaction, 0.30 g of yellow *cis*-$[PtCl_2(NH_3)_2]$ (0.10 mmol) was suspended in a solution of 0.074 g KCl (0.10 mmol) in 25 mL of distilled water, and the mixture, protected from light by aluminum foil, was stirred, while nitrogen dioxide was bubbled through the solution at a rate of approximately 1 bubble per second. The cylinder of NO_2 was warmed in a water bath at ~45 °C to increase the vapor pressure of the gas (bp = 21.1 °C) to a useful level.

In most cases, an initial color change of the reaction mixture to light cloudy green was immediately noted, and within approximately 1–2 min, the suspension dissolved and formed a dark turquoise solution. Once this change was observed, the NO_2 gas flow was terminated, and breathing quality air was bubbled through the stirred solution overnight, while the solution changed gradually from green to pale yellow. The following day, additional NO_2 gas was bubbled briefly through the solution; if the solution remained yellow upon the addition of the gas, the reaction was considered to have gone to completion, and the mixture was pumped down to dryness at reduced pressure (10^{-2} torr). If, however, the solution turned green upon the addition of further NO_2, bubbling of air was continued until the solution turned yellow, at which point the water was removed at reduced pressure as above. The yellow products obtained in the two procedures appeared to be identical.

This synthetic route was carried out several times to give 0.33–0.37 g of pale yellow powders, which upon dissolution in acetone give yellow solutions, and a white, insoluble material, which was not identified but is presumably a potassium byproduct. The powders exhibit slight solubility in water, dimethylsulfoxide, and dimethylformamide; aqueous solutions are stable for a few hours, and DMSO and DMF solutions are stable for several days.

A few small yellow crystals were grown by slow evaporation of an acetone solution of the compound, and an X-ray crystal structure determination of one of these was performed by Dr. Ruiyao Wang in the X-ray Crystallography Laboratory at Queen's University. A crystal of the compound (light yellow, plate-shaped, size 0.08 × 0.06 × 0.03 mm) was mounted on a glass fiber with grease and cooled to −93 °C in a stream of nitrogen gas controlled with Cryostream Controller 700. Data collection was performed on a Bruker SMART APEX II X-ray diffractometer with graphite-monochromated Mo Kα radiation ($\lambda = 0.71073$ Å), operating at 50 kV and 30 mA over 2θ ranges of 7.34−53.90°. No significant decay was observed during the data collection.

Data were processed on a PC using the Bruker AXS Crystal Structure Analysis Package:[4a] data collection, APEX2 (Bruker, 2006); cell refinement, SAINT (Bruker, 2005); data reduction, SAINT (Bruker, 2005); structure solution, XPREP (Bruker, 2005) and SHELXTL (Bruker, 2000); structure refinement, SHELXTL; molecular graphics, SHELXTL; publication materials, SHELXTL. Neutral atom scattering factors were taken from Cromer and Waber.[4b] The crystal was the orthorhombic space group *Cmcm*, based on the systematic absences, *E* statistics, and successful refinement of the structure. The structure was solved by direct methods. Full-matrix least-squares refinements minimizing the function $\Sigma w(F_o^2 - F_c^2)^2$ were applied to the compound. All nonhydrogen atoms were refined anisotropically. The positions for all hydrogen atoms were calculated, and their contributions were included in the structure factor calculations. Convergence to final $R_1 = 0.0218$ and $wR_2 = 0.509$ for 359 ($I > 2\sigma(I)$) independent reflections, and R1 = 0.0323 and wR2 = 0.0604 for all 456 (R(int) = 0.0482) independent reflections, with 36 parameters and 6 restraints, were achieved. The largest residual peak and hole was found to be 1.647 and −0.695 e/Å,^{3} respectively.

The crystal contained equal amounts of *fac*- and *mer*-$[PtCl_3(NO_2)(NH_3)_2]$ (see discussion of crystal structure below). Selected bond lengths and angles are provided in Table 1, general crystallographic data in Table 2, and the molecular structures are

(3) (a) Yu, H.; Jove, R. *Nat. Rev. Cancer* **2004**, *4*, 97. (b) Bromberg, J. F.; Wrzeszczynska, M. H.; Devgan, G.; Zhao, Y.; Pestell, R. G.; Albanese, C.; Darnell, J. E., Jr. *Cell* **1999**, *98*, 295. (c) Niu, G.; Wright, K. L.; Huang, M.; Song, L.; Haura, E.; Turkson, J.; Zhang, S.; Wang, T.; Sinibaldi, D.; Coppola, D.; Heller, R.; Ellis, L. M.; Karras, J.; Bromberg, J.; Pardoll, D.; Jove, R.; Yu, H. *Oncogene* **2002**, *21*, 2000. (d) Anagnostopoulou, A.; Vultur, A.; Arulanandam, R.; Cao, J.; Turkson, J.; Jove, R.; Kim, J. S.; Glenn, M.; Hamilton, A. D.; Raptis, L *Cancer Lett.* **2006**, *242*, 120.

(4) (a) *Bruker AXS Crystal Structure Analysis Package: SHELXTL*, version 6.14; *XPREP*, version 200 5/2; *SAINT*, version 7.23A; *APEX2*, version 2.0–2; Bruker AXS Inc.: Madison, WI, 2000–2006. (b) Cromer, D. T.; Waber, J. T. *International Tables for X-ray Crystallography*; Kynoch Press: Birmingham, U.K., 1974; Vol. *4*, Table 2.2A.

(5) (a) Decker, S. J. *J. Biol. Chem.* **1989**, *264*, 17641. (b) Vultur, A.; Arulanandam, R.; Turkson, J.; Niu, G.; Jove, R.; Raptis, L. *Mol. Biol. Cell* **2005**, *16*, 3832. (c) Turkson, J.; Bowman, T.; Garcia, R.; Caldenhoven, E.; de Groot, R. P.; Jove, R. *Mol. Cell. Biol.* **1998**, *18*, 2545. (d) Turkson, J.; Ryan, D.; Kim, J. S.; Zhang, Y.; Chen, Z.; Haura, E.; Laudano, A.; Sebti, S.; Hamilton, A. D.; Jove, R. *J. Biol. Chem.* **2001**, *276*, 45443.

Table 1. Selected Bond Lengths for *fac*-[$PtCl_3(NO_2)(NH_3)_2$]

bond	bond length (Å)
Pt(1)−Cl(3)	2.278(5)
Pt(1)−Cl(3B)	2.278(5)
Pt(1)−N(3A)	2.05(3)
Pt(1)−N(3C)	2.05(3)
Pt(1)−N(1)	2.04(2)
Pt(1)−Cl(1)	2.346(5)

Table 2. Crystal Data and Structure Refinement for [$PtCl_3(NO_2)(NH_3)_2$]

empirical formula	$H_6Cl_3N_3O_2Pt$
fw	381.52
temp	180(2) K
wavelength	0.71073 Å
cryst syst	Orthorhombic
space group	*Cmcm*
unit cell dimensions	$a = 6.545(3)$ Å
	$b = 10.508(4)$ Å
	$c = 10.627(4)$ Å
	$\alpha = 90°$
	$\beta = 90°$
	$\gamma = 90°$
vol	730.9(5) Å^3
Z	4
density (calcd)	3.467 Mg/m^3
abs coefficient	20.229 mm^{-1}
F(000)	688
cryst size	0.08 × 0.06 × 0.03 mm^3
θ range for data collection	3.67−26.95°
index ranges	$-8 \le h \le 8$
	$-13 \le k \le 13$
	$-13 \le l \le 13$
reflns collected	3624
independent reflns	456 [*R*(int) = 0.0482]
completeness to $\theta = 26.95°$	100.0%
abs correction	multiscan
max. and min. transm	0.5821 and 0.2945
refinement method	full-matrix least-squares on F^2
data/restraints/params	456/6/36
GOF on F^2	1.000
Final R indices [$I > 2\sigma(I)$]	R1 = 0.0218, wR2 = 0.0509
R indices (all data)	R1 = 0.0323, wR2 = 0.0604
largest diff. peak and hole	1.647 and −0.695 e Å^{-3}

shown in Figure 5. Full crystallographic data, atomic coordinates and equivalent isotropic displacement parameters, bond lengths and angles, anisotropic displacement parameters, hydrogen coordinates and isotropic displacement parameters, and torsion angles are given in the Supporting Information.

Examination of Stat, tyr-705 Phosphorylation and Transcriptional Activity. NIH3T3 cells overexpressing the human EGF receptor (hEGFR)[5a] were grown in plastic dishes in DMEM supplemented with 10% calf serum, in a 5% CO_2 incubator. All Stat3 activity measurements were conducted at a cell confluence of 50%, which was estimated visually and quantitated by imaging analysis of live cells under phase contrast using a Leitz Diaplan microscope and the MCID-elite software (Imaging Research, St. Catharine's,Ontario) CPA-7 and $PtCl_4$ were dissolved in 50% DMSO at concentrations of 20 mM and stored at −80 °C in aliquots. Both are stable in this form for up to a year. They were thawed immediately before use and were added directly to the growth medium at the final concentrations indicated (Figure 6). Following drug treatment, the EGF receptor was activated by the addition of 100 μg/mL EGF for 10 min.

For the examination of Stat3-ptyr705 phosphorylation, total cellular protein was extracted using 50 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 0.5 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1% Triton X-100;[5b] 50 μg of clarified cell extract protein were resolved on a 10% polyacrylamide-SDS gel and transferred to a nitrocellulose membrane (Bio-Rad). The membranes were blocked with 5% nonfat milk for at least one hour, followed by an overnight incubation in primary antibody against the tyrosine-705 phosphorylated, that is, activated form of Stat3 (Biosource), or against the dually phosphorylated, that is, activated form of Erk1/2 (Biosource), followed by alkaline phosphatase-conjugated goat secondary antibodies (Biosource). The bands were visualized using enhanced chemiluminescence (ECL), according to the manufacturer's instructions (Perkin-Elmer Life Sciences, catalog no. NEL602). As a control for protein loading, blots were probed with a mouse monoclonal anti-Hsp90 antibody (Stressgen), followed by a secondary antibody and ECL detection as above. Quantitation was achieved by fluorimager analysis using the FluorChem program (AlphaInnotech Corp).

For the examination of the effect of CPA-7 upon Stat3 transcriptional activity, we used vSrc-transformed NIH3T3 cells, transfected with a Stat3-specific reporter plasmid (p*Luc*TKS3) which harbors seven copies of a sequence corresponding to the Stat3-specific binding site in the C-reactive gene promoter (termed APRE, TTCCCGAA) upstream from a firefly luciferase coding sequence.[5c]

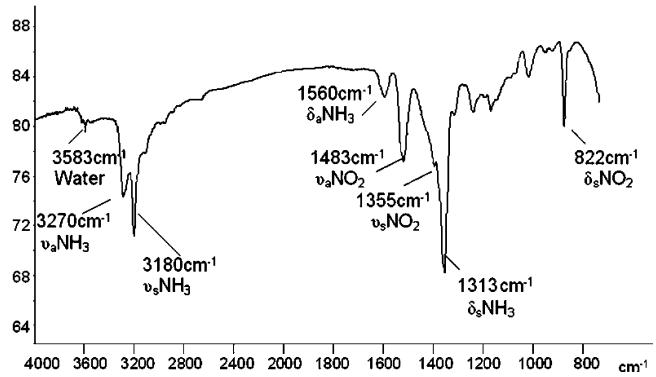

Figure 1. IR spectrum of a representative sample of [$PtCl_3(NO_2)(NH_3)_2$].

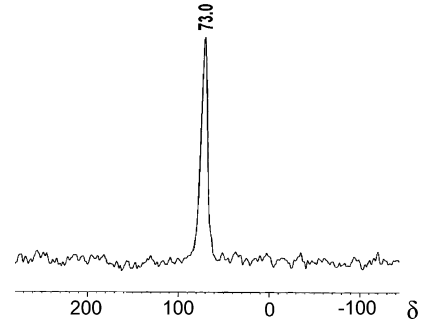

Figure 2. Representative ^{195}Pt NMR spectrum of [$PtCl_3(NO_2)(NH_3)_2$].

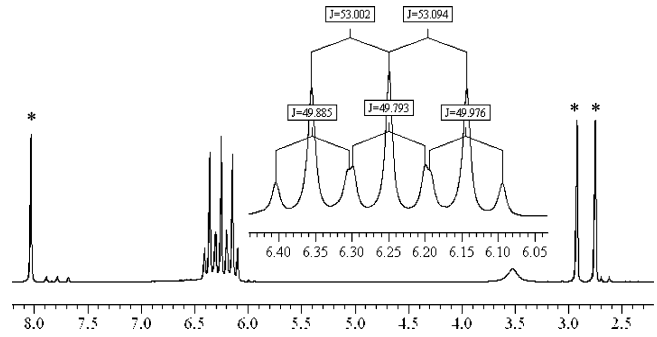

Figure 3. ^{1}H NMR spectrum (in DMF-d_7) of [$PtCl_3(NO_2)(NH_3)_2$]: the broad resonance at $\delta \sim 3.5$ is attributed to water; those marked with asterisks are attributed to residual protons in the solvent.

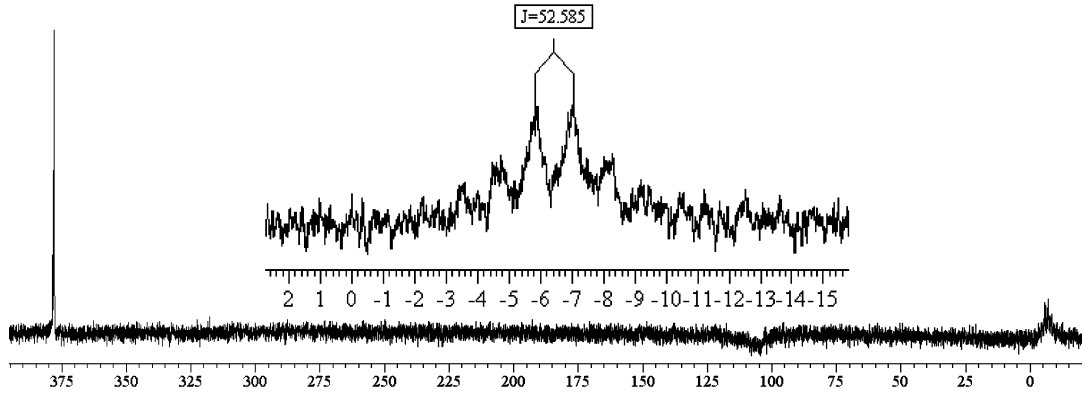

Figure 4. ^{14}N NMR spectra of $[PtCl_3(NO_2)(NH_3)_2]$ (DMSO-d_6).

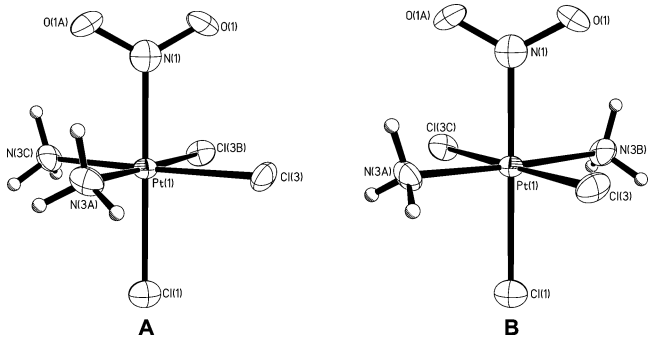

Figure 5. Structures of (A) the *fac*-isomer and (B) the *mer*-isomer of $[PtCl_3(NO_2)(NH_3)_2]$.

As a control, p*Luc*TKS3-expressing cells were stably transfected with a different reporter, pRLSRE, which contains two copies of the serum response element (SRE) of the c-fos promoter, subcloned into the *Renilla* luciferase reporter, pRL-null (Promega), and Zeocin-resistance coselection.[5d] The firefly and *Renilla* luciferases use different substrates and thus can be assayed independently in the same lysates (Promega). After they were treated for 24 h, the cells were lysed, and the luciferase activity was determined.

Results and Discussion

Synthesis and Structural Characterization of $[PtCl_3(NO_2)(NH_3)_2]$ (CPA-7). Useful amounts (0.3–0.4 g) of powdery substances of the correct color (light yellow) and exhibiting useful activities as Stat3 inhibitors (see subsection below, Assessment of Biological Activities) were successfully obtained using a procedure adapted from a recent patent.[2c] Although the workup procedure resulted in the crude products containing an unidentified potassium-containing byproduct and all attempts to obtain analytically pure materials resulted in decomposition, we are confident that we have produced the substance used elsewhere as CPA-7 in Stat3 inhibitor drug studies.[2]

We note at this point that one or more compounds of the formula $[PtCl_3(NO_2)(NH_3)_2]$ have been reported earlier, in the 1960s.[6] For instance, Chernyaev et al.[6a–c] reported compounds identified as such, but the synthetic procedures are not described in sufficient detail to be repeatable and none of the products was characterized in a manner which is unambiguous or definitive. During the same period, Le Postollec reported IR spectra of hexachlorobutadiene mulls of a series of platinum compounds including one said to be $[PtCl_3(NO_2)(NH_3)_2]$,[6d] but it is not possible to deduce how the compound was made or on what basis it was identified. Somewhat later, in 1974, Samatov et al.[6e] described $[PtCl_3(NO_2)(NH_3)_2]$ to be a yellow, light-sensitive, acetone-soluble compound, but again, the synthetic procedure is not amenable to repetition, and no spectroscopic properties useful for purposes of comparison were reported. However, because $[PtCl_3(NO_2)(NH_3)_2]$ was said to be light sensitive,[6e] we carried out as much of our work as possible with the substances protected by aluminum foil.

Because CPA-7 is a very potent anticancer drug, the biology of $[PtCl_3(NO_2)(NH_3)_2]$ has more recently been investigated in great detail but again there is a paucity of *chemical* information concerning its synthesis and characterization.[2] For instance our synthetic procedure had to be adapted from a more general methodology apparently applicable to a range of Pt(IV) compounds, and we could find no spectroscopic or crystallographic data which one could use to establish the identity, structure, or purity of CPA-7. With a view toward establishing criteria by which CPA-7 could be recognized by researchers in this field, we have therefore not only developed a reproducible synthetic methodology for CPA-7, but we have also obtained a reasonably complete set of structural information data: IR spectroscopy, ^{1}H, ^{14}N, ^{15}N, and ^{195}Pt NMR spectroscopy, and single crystal X-ray crystallography.

A typical IR spectrum of a solid sample is shown in Figure 1 and clearly exhibits the absorptions characteristic of a N-coordinated nitro group (ν(NO) = 1483, 1355 cm^{-1}, δ(ONO) = 822 cm^{-1})[7a] and coordinated NH_3 ($\nu(NH_3)$ = 3270, 3180 cm^{-1}, $\delta(NH_3)$ = 1560, 1313 cm^{-1}).[7b] Thus the substances obtained all contained these two ligands, although

(6) (a) Chernyaev, I. I.; Muraveiskaya; G. S.; Korablina, L. S. *Russ. J. Inorg. Chem.* **1965**, *10*, 158. (b) Chernyaev, I. I.; Muraveiskaya, G. S.; Korablina, L. S. *Russ. J. Inorg. Chem.* **1966**, *11*, 728. (c) Chernyaev, I. I.; Leonova, T. N. *Russ. J. Inorg. Chem.* **1969**, *14*, 307. (d) Le Postollec, M. *J. Chim. Phys Phys.-Chim. Biol.* **1965**, *62*, 67. (e) Samatov, A. G.; Zheligovskaya, N. N.; Spitsyn, V. I. *Bull. Acad. Sci, USSR, Ser. Chem.* **1974**, 1390.

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these IR data do not provide definitive information concerning the stoichiometry or structure, of course. The IR spectra often exhibit a weak, broad band at ~3580 cm^{-1}, attributable to the presence of water.

Several of the products obtained were also characterized by ^{195}Pt NMR spectroscopy.[8] As an analytical tool ^{195}Pt NMR spectroscopy is very useful because of the enormous chemical shift range for this nucleus, up to 13 000 ppm depending on the oxidation state and the ligands present.[8] Thus even minor changes in the inner coordination spheres of a series of similar compounds can result in measurable differences in ^{195}Pt chemical shifts, and we anticipated that this spectroscopic technique would not only readily be informative concerning the presence of multiple products but also concerning the hydrolytic stability of $[PtCl_3(NO_2)(NH_3)_2]$ itself.

Figure 2 shows a ^{195}Pt NMR spectrum of a representative, freshly prepared sample in D_2O. As can be seen, there is only a single, symmetric resonance at δ 73.0, broadened somewhat ($\Delta\nu_{1/2} \approx 1000$ Hz) by chemical shift anisotropy effects.[8] This result is quite reproducible and is indicative of the formation of only a single product which is, on the basis of the chemical shift, a compound of Pt(IV).[8] The spectrum did not change upon the mixture standing at room temperature for at least 48 h, suggesting that the species in solution is very stable because the ^{195}Pt NMR spectrum of a mixture of presumably similar materials obtained by reacting *cis*-$[PtCl_4(NH_3)_2]$ with $AgNO_2$ (see below) exhibited a much broader, asymmetric resonance.

A representative ^{1}H NMR spectrum of a freshly prepared sample in DMF-d_7 is shown in Figure 3. The ammine resonance at δ 6.25 exhibits remarkably narrow lines because of the near cubic symmetry of the ^{14}N nucleus,[9a] and is observed as a symmetric 1:1:1 triplet of 1:4:1 triplets with $J(^1H-^{14}N) = 53.0$ Hz, $J(^1H-^{195}Pt) = 49.9$ Hz. The six ammine hydrogens are thus all equivalent which means that the two ammine ligands are positioned in identical environments, consistent with the *fac*-isomer shown above. A weak triplet is also observed at δ 7.80, but there is no coupling to ^{195}Pt, and thus the resonance is presumably to be attributed to an ammonium salt contaminant.

A ^{14}N NMR spectrum of a representative sample was also run and found to be consistent with the presence of a single compound in solution, for example, the *fac* structure of CPA-7 as described above. As can be seen in Figure 4, two resonances were observed, a sharp singlet at δ 378 and a broad quartet at δ −6.5. The former is readily assigned to the nitro group,[10a] the latter to the ammine ligands,[10] for which $J(^1H-^{14}N) \approx 53$ Hz, as in the ^{1}H NMR spectrum. The $^{14}N-^{195}$Pt coupling was not observed because of the low signal/noise ratio, but would be expected to be in the range of 170–195 Hz.[10c,d] Consistent with this prediction, given that the ratio of the ^{15}N and ^{14}N gyromagnetic ratios is ~1.40,[9b] a complementary 2D $^1H-^{15}N$ HSQC experiment found $J(^{15}N-^{195}Pt)$ to be ~260 Hz.

Significantly, an attempt to prepare CPA-7 via chloride abstraction from *cis*-$[PtCl_4(NH_3)_2]$ with $AgNO_2$ in water

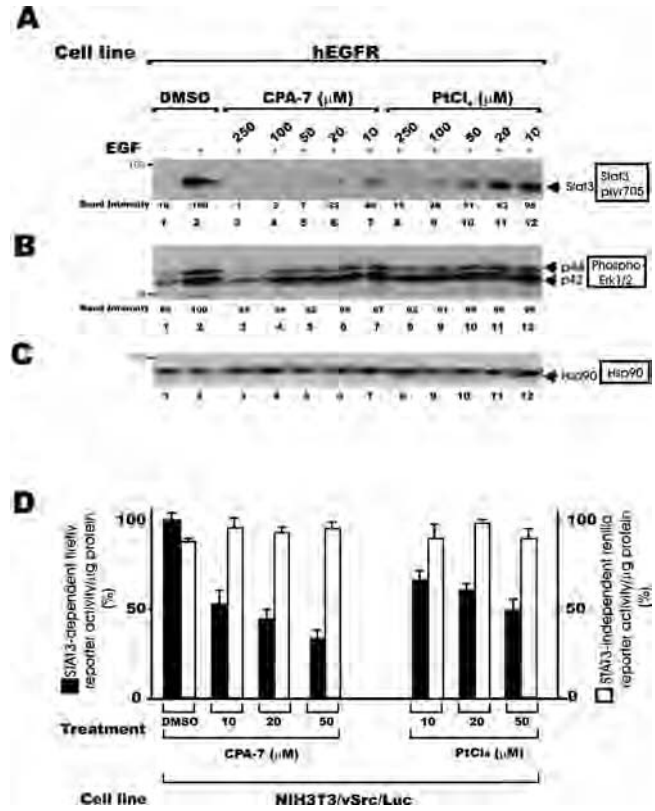

Figure 6. A−C: CPA-7 can inhibit Stat3-tyr705 phosphorylation. Mouse NIH3T3 fibroplasts overexpressing the human EGF receptor (hEGFR) were grown to 50% confluence and treated with CPA-7 (lanes 3–7) or $PtCl_4$ (lanes 8–12), or the DMSO carrier alone (lanes 1,2) for 24 h. Cells were subsequently stimulated with 100 ng/mL EGF for 10 min. Blots of detergent extracts of total protein were probed with an antibody against the tyr-705 phosphorylated form of Stat3 (A), Erk1/2 (B), or Hsp90 as a loading control (C), as indicated. Numbers at the left correspond to molecular weight markers. Numbers under the lanes in panels (A) and (B) refer to band intensities obtained through quantitation by fluorimager analysis and normalized to Hsp90 levels, with the peak value of the control, DMSO-treated, EGF-stimulated cells (lane 2) taken as 100% (see Experimental Section). Arrows point to the position of Stat3, Erk1/2, or Hsp90, respectively, as indicated. D: CPA-7 can inhibit Stat3 transcriptional activity. NIH3T3 cells stably expressing vSrc and the Stat3-dependent p*Luc*TKS3 reporter driving a firefly luciferase gene, and the Stat3-independent pRLSRE reporter driving a *Renilla* luciferase gene under control of the c-fos SRE promoter, respectively (vSrc/NIH3T3/Luc cells) were grown to 50% confluence and firefly (■) and Renilla (□) luciferase activities determined in cytosolic extracts following CPA-7 or $PtCl_4$ treatment, as indicated (see Experimental Section). Values shown represent luciferase units expressed as percent of the highest value obtained, means plus standard deviations of at least three experiments, each performed in triplicate.

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resulted in the formation of a yellow substance, for which the IR spectrum indicated the presence of nitro and ammine ligands. The ^{1}H NMR spectrum (DMF-d_7), however, exhibited at least two sets of ammine multiplets in the region δ 5.8–6.5, while the ^{195}Pt NMR spectrum exhibited an asymmetric resonance centered at δ 72.4 which was 3–4 times as broad as that described above for freshly prepared CPA-7 (Figure 2). Therefore, while the silver ion did abstract chloride ions concomitantly with nitrite coordination, we clearly obtained a mixture of isomers, compounds, or both. While this experiment was unsuccessful, it did validate our use of ^{1}H and ^{195}Pt NMR spectroscopy to conclude that freshly prepared CPA-7 was obtained as a single isomer.

Although we were unable to obtain analytically pure product in bulk, an X-ray quality crystal was acquired by allowing a solution in acetone to evaporate slowly to near dryness in the dark. The crystal failed to refine satisfactorily for the anticipated *fac* structure of $[PtCl_3(NO_2)(NH_3)_2]$, but subsequent refinement found a better and more stable solution in which equal amounts of *fac*- and *mer*-$[PtCl_3(NO_2)(NH_3)_2]$ were present. The structure is a good example of so-called "whole molecule disorder" in which the chloride ligands can appear on any of the six vertices of the coordination octahedron (Figure 1 of Supporting Information, in which the ammine and chloride ligands are positioned in the $PtCl_2N_2$ plane, nitro and chloride ligands above and below the plane). Crystallographically, the four positions in the $PtCl_2N_2$ plane are equal, but the two positions above and below the plane are not. Each planar position is occupied by chloride or ammine ligands with 50% probabilities of either, but the nitro and the chloride ligands that are not in the plane are in two parts, groups 1 and 2. The major portion is group 1, labeled as N(1)O(1)(O1A) and Cl(1), which occurs in ~79% of the molecules. The minor part is group 2, labeled as N(2)O(2)O(2A) and Cl(2), which occurs in ~21% of the molecules.

Selected bond distance data are given in Table 1. As a result of the disorder, the Pt−NH_3 distances, 2.05(3) Å, were not determined to a high degree of accuracy. Relevant published data with which comparisons may be made are 2.01(4) Å for *cis*-$[PtCl_2(NH_3)_2]$ (cisplatin),[11a] 2.05(4) Å for *trans*-$[PtCl_2(NH_3)_2]$,[11a] and 2.069(6) and 2.064(7) Å for *trans*-$PtCl_4(NH_3)_2$ cocrystallized with 1-methyluracil,[11b] and thus the result for $[PtCl_3(NO_2)(NH_3)_2]$ is reasonable. The in-plane Pt−Cl distances are 2.278(5) Å, significantly shorter than in *cis*-$[PtCl_2(NH_3)_2]$, *trans*-$[PtCl_2(NH_3)_2]$ and *trans*-$[PtCl_4(NH_3)_2]$, 2.33(1), 2.32(1), and 2.3137(10) Å, respectively. In contrast, the Pt−Cl bond trans to the nitro ligand is significantly longer (2.346(5) Å) because of the relatively high trans influence of the nitro ligand.[12] The Pt−NO_2 bond distance of 2.04(2) Å is very similar to the average of the Pt−NO_2 distances in *trans*-dichlorodinitroethylenediamineplatinum(IV), 2.09 Å.[11c]

Unfortunately, given the time required to grow the crystal used and the fact that it turned out to be a mixture of isomers, the crystalline sample was apparently not representative of the freshly prepared crude samples of CPA-7, for which all spectroscopic data are consistent only with the presence of a single species.

The mechanism by which *cis*-$[PtCl_2(NH_3)_2]$ is converted to *fac*-$[PtCl_3(NO_2)(NH_3)_2]$ (CPA-7) via reaction with NO_2 in the presence of air has not been elucidated. Compounds of platinum(II) are normally quite stable with respect to oxidation, and oxidation procedures normally involve use of strong oxidants such as hydrogen peroxide or chlorine.[14] By analogy with the chemistry of ethylenediamine complexes of platinum(II),[15] the chemistry involved in the synthesis of CPA-7 is probably quite complex.

Assessment of Biological Activities. Following the synthetic investigation, the biological activities of several samples were examined by Dr. Leda Raptis' laboratory at Queen's University and compared to that of $PtCl_4$. Because the activity of Stat3 was previously shown to be affected by cell to cell adhesion,[13] the effects of CPA-7 and $PtCl_4$ upon Stat3 activity were examined at a confluence of 50%. NIH3T3 cells overexpressing the human EGF receptor (hEGFR cells) were plated in 3 cm Petri dishes and treated with different concentrations of the drugs for 24 h, followed by activation of the EGFR by EGF addition. Proteins in detergent cell extracts were subsequently probed for Stat3-ptyr705 (see Experimental Section). As shown in Figure 6A, there was a dramatic reduction in Stat3-ptyr705 levels at CPA-7 concentrations of greater than 50 μM (lane 5 vs lane 2), while at 10 μM there was still a 5-fold decrease. $PtCl_4$ was less effective, with a comparable reduction at concentrations of greater than 100 μM (lane 9).

As a control for specificity of inhibition of Stat3, the extracts were blotted against the dually phosphorylated, that is, activated form of an unrelated kinase, the extracellular signal regulated kinase (Erk1/2). As shown in Figure 6B, CPA-7 treatment had no effect upon Erk1/2 activity at concentrations of up to 100 μM, although at 250 μM, there was a reduction of approximately 2-fold. $PtCl_4$ treatment had no measurable effect upon Erk1/2 levels at any of the concentrations tested.

To further examine the effect of these compounds upon Stat3 transcriptional activity, we conducted luciferase assays using vSrc-transformed cells expressing a luciferase gene construct under control of a Stat3-specific promotor (p*Luc*TKS3 plasmid, see Experimental Section). Because of the presence of the vSrc oncogene, these cells have constitutively high luciferase activity. Cells were treated with different concentrations of the compounds (10−50 μM) for 24 h, followed by protein extraction and measurement of luciferase activity in detergent cell extracts. As shown in Figure 6D, treatment with 50 μM CPA-7 caused ap-

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proximately a 60% reduction in activity, while the same concentration of $PtCl_4$ caused approximately a 40% reduction. The residual activity could be the result of the endogenous luciferase present in these cells, which had not had sufficient time to decay during the 24 h of treatment. On the other hand, Stat3-independent transcription from the c-fos, SRE promotor element was not affected by CPA-7, indicating that this compound inhibits Stat3 activity specifically. The above data taken together indicate that, aside from inhibition of Stat3-ptyr705 phosphorylation, CPA-7 can also inhibit Stat3 transcriptional activity.

Conclusions. A simple, reproducible method of synthesizing the potentially useful anticancer agent, *fac*-$[PtCl_3(NO_2)(NH_3)_2]$ (CPA-7), has been developed. The synthesis involves the conversion of *cis*-$[PtCl_2(NH_3)_2]$ (cisplatin) via oxidation by nitrogen dioxide in the presence of oxygen and chloride ion. The product was fully characterized by spectroscopic and crystallographic data, and shown to behave as an effective Stat3 inhibitor.

Supporting Information Available: Crystallographic details, including figures of $[PtCl_3(NO_2)(NH_3)_2]$ showing complete numbering schemes and thermal ellipsoid figures, and tables of positional and thermal parameters and bond lengths and angles. This material is available free of charge via the Internet at http://pubs.acs.org.

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