# A Tetranuclear Platinum Compound Designed to Overcome Cisplatin Resistance

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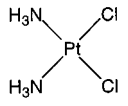
The synthesis and characterisation of the first generation of a poly(propyleneimine) dendrimer DAB(PA)$_4$, substituted with four *trans*-diamminechloroplatinum moieties is reported. The compound DAB(PA-tPt-Cl)$_4$ was designed to overcome two problems often associated with cisplatin resistance in cancer cells: (i) deactivation of the platinum species by intracellular thiolates and (ii) improved repair of crosslinks with DNA. The four-armed molecule can be expected to form crosslinks with DNA that are very different from the adducts formed by cisplatin. Also, the tetranuclear compound has four leaving groups, while cisplatin has only two. Therefore, DAB(PA-tPt-Cl)$_4$ would be less susceptible towards inactivation by reaction with intracellular thiolates. A reaction with an excess of the model nucleobase guanosine 5′-monophosphate (GMP) confirmed that the tetranuclear compound is capable of binding a maximum of four nucleobases. Therefore, the inactivation of one or two arms would still leave the molecule with enough reactivity to form crosslinks with DNA. Cytotoxicity tests were performed on two mouse leukemia L1210 cell lines, both sensitive and resistant towards cisplatin, and in seven human tumor cell lines. In all cell lines, the tetranuclear compound showed a low cytotoxicity. It is suggested that the low activity is related to the structure of the compound. Probably the high charge (+6) at physiological pH and its branched structure hamper the molecule in crossing the cell membranes.

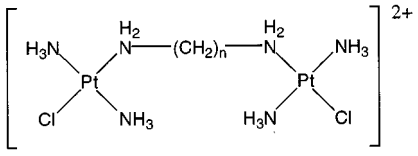
## Introduction

Since the discovery of cisplatin as antitumor drug, much effort has been undertaken to find new platinum compounds applicable as a drug against cancer.[1−3] One of the main objectives in platinum antitumor chemistry is to find compounds with activity against cancer cells having intrinsic or acquired resistance against cisplatin.[3] It is now generally accepted that the antitumor activity of cisplatin stems from the complex events that occur after its binding to DNA.[1−3] Known mechanisms for cisplatin resistance include improved recognition of DNA adducts by DNA repair mechanisms, decreased drug accumulation in the cell (due to decreased influx) and increased intracellular levels of the heavy-metal scavengers glutathione (GSH) and metallothionein (MT).[4−6]

Many derivatives with structures similar to cisplatin were found to be cross resistant with cisplatin. One approach to overcome cisplatin resistance is the preparation of drugs that bind to DNA in a structurally different way compared to cisplatin.[1] In this respect, the dinuclear 1,1/t,t platinum complexes,[7][8] with the general formula [{*trans*-PtCl(NH$_3$)$_2$}μ-{NH$_2$(CH$_2$)$_n$NH$_2$}](NO$_3$)$_2$ ($n$ = 4, 6), are very interesting (Scheme 1). These compounds consist of two monofunctional [PtCl(NH$_3$)$_2$] moieties connected by a flexible diamine linker. This type of compounds was found to be active against cisplatin resistant cancers in vivo.[8] The striking difference in DNA binding behavior of such compounds is illustrated by the hairpin structure of an interstrand adduct of 1,1/t,t ($n$ = 4) with a dsDNA octamer.[9] Binding of cisplatin to DNA is mainly restricted to intrastrand crosslinks between adjacent purine bases, with only 5−10% interstrand crosslinks.[10][11] In contrast, the number of interstrand crosslinks is reported to be much larger (50%) for dinuclear platinum compounds.[12]




Cisplatin




1,1/t,t (n=4, 6)

Scheme 1. Cisplatin and a dinuclear compound of the 1,1/t,t type

Recently, the diaminoalkyl linker was replaced by the naturally occurring polyamines spermine and spermidine.[13] The protonated, noncoordinating secondary amines in these molecules may have an additional interaction with

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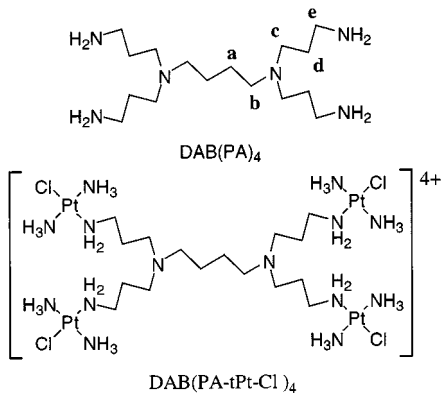
DNA by hydrogen bonding and electrostatic interaction. At the moment, the most promising variation appears to be the bifunctional trinuclear compound BBR 3464 [μ-*trans*-$Pt(NH_3)_2\{transPtCl(NH_3)_2[NH_2(CH_2)_6NH_2]\}_2](NO_3)_4$.[14][15] This cationic compound can be seen as a linear molecule with two ends capable of DNA binding, and a central platinum moiety that is restricted to noncovalent interactions. BBR 3464, with a total charge of +4, has now entered clinical trials.[14]

It has been suggested that the increased number of interstrand crosslinks is the reason for the high activity of dinuclear compounds against cisplatin-resistant cell lines.[12] From this point of view, it is interesting to investigate variatons on the dinuclear 1,1/t,t compounds with a number of DNA-binding moieties higher than two. One way to obtain more DNA-binding moieties is to increase the number of platinated "arms". A dinuclear molecule may be considered as having two arms. Statistically, additional arms should increase the number of interstrand adducts formed compared to dinuclear compounds. An additional advantage of this type of compound could be that after deactivation of one, or even two, of its arms by GSH or MT, the molecule still retains enough active arms left to form a bifunctional adduct with DNA.

In this paper, the synthesis and characterisation is reported of a novel tetranuclear platinum compound $DAB(PA\text{-}tPt\text{-}Cl)_4$ based on the first generation of the commercially available butanediamine poly(propyleneimine) dendrimer $DAB(PA)_4$ (Scheme 2). This first generation dendrimer provides four amine end-groups that can be functionalized by platinum. The structure was characterized by $^{195}Pt$ NMR, elemental analysis, and a pH titration followed by $^1H$ NMR spectroscopy. The reactivity of the platinated arms towards DNA was checked by a test reaction with guanosine monophosphate (GMP), monitored by $^1H$ NMR. The in vitro cytotoxicity was tested on L1210 mouse leukemia cell lines, including cell lines resistant towards cisplatin. As a reference, cisplatin was tested as well.




Scheme 2. The first generation poly(propyleneimine) dendrimer $DAB(PA)_4$, and the tetranuclear platinum complex $DAB(PA\text{-}tPt\text{-}Cl)_4$

## Results and Discussion

### Synthesis and Characterisation

For the preparation of the dendritic platinum complex $DAB(PA\text{-}tPt\text{-}Cl)_4$, a variation on the common synthesis of dinuclear compounds was applied.[16] The polyamine was reacted with an excess of the monoactivated solvent species *trans*-$[PtCl(NH_3)_2(dmf)]NO_3$. The chloride salt of the tetranuclear complex was obtained by quenching the reaction with an excess of LiCl. The excess of LiCl is later removed by extraction with ethanol or methanol. *trans*-$[PtCl_2(NH_3)_2]$ is easily separated from the product, while it has a low solubility in water, in contrast to $(PA\text{-}tPt\text{-}Cl)_4$.

$^{195}Pt$ shows a single signal at $\delta = -2425$, which is identical to that of the $[PtClN_3]$ coordination sphere found for the dinuclear 1,1/t,t compounds.[17] The two tertiary amine atoms present in $DAB(PA)_4$ are potential coordination sites for platinum, although they are poorer donors than the four primary amines. In order to confirm the coordination site, pH titrations of the complex and the free ligand were followed by $^1H$ NMR spectroscopy (Figure 1). The spectrum shows shifted peaks and a different pH dependence compared to the free ligand.

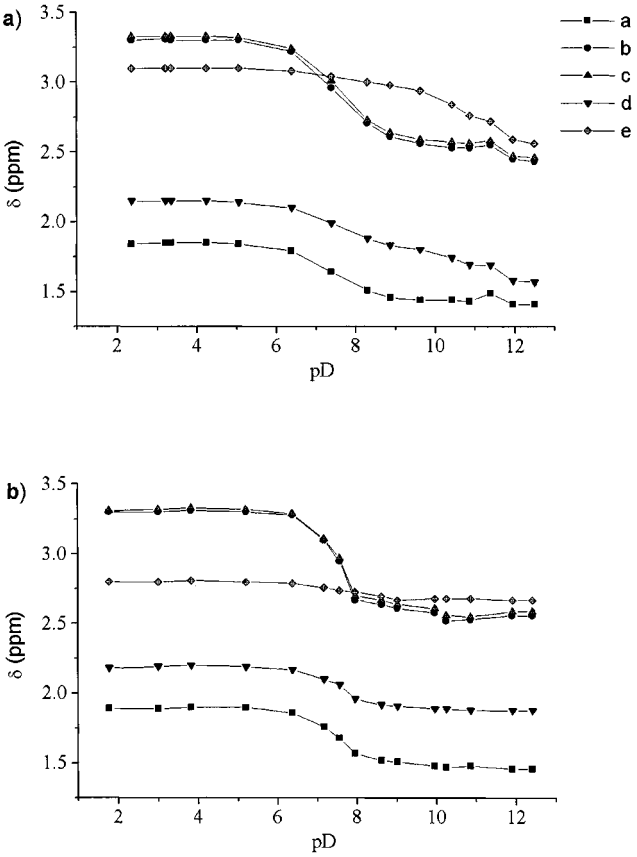



Figure 1. Titration curves of the proton signals of a) the ligand $DAB(PA)_4$ and b) the platinum complex $DAB(PA\text{-}tPt\text{-}Cl)_4$

The titration curve of $DAB(PA)_4$ is in general agreement with the data recently determined for this compound using natural abundant $^{15}N$ spectroscopy.[18] Upon lowering the

pH, first the primary and then the tertiary nitrogens are protonated. The protons shifted most upfield show only small pH dependence and are identified as protons **a** and **d** (Scheme 2). Protons **b** and **c** show overlapping signals at all pH values, and are identified by their total integral value of 12 protons. The remaining peak is assigned to **e**. The assignment was confirmed by a [$^1$H,$^1$H] COSY NMR experiment. Metalation should protect the amines from protonation. In the titration of the complex, pH dependence of the shift of proton **e** is only marginal compared to its behavior in the free ligand. In contrast protons **b** and **c** show a strong pH dependence in both titrations. This allows the conclusion that platinum is coordinated at the primary amines. At physiological pH, the tertiary amines are hydronated, thus giving the molecule a total charge of +6. This high positive charge is likely to give strong coulomb interactions with a polyanion like DNA.

## Reaction with GMP, a Model Nucleobase

The coordination of DAB(PA-tPt-Cl)$_4$ with the model nucleobase guanine-5′-monophosphate (GMP) was studied by $^1$H NMR spectroscopy. The reaction was performed in $D_2O$ at 313 K, with a molar ratio of 5:1 ([GMP]:[DAB(PA-tPt-Cl)$_4$]). Results are summarized in Table 1. Signals of a platinated product appeared immediately after mixing the two compounds. After four days the reaction was judged to be complete. The single signal in $^{195}$Pt NMR at $\delta = -2560$ proves that all platinum has reacted. Also, the ratios of platinated and free GMP were found constant. This ratio was determined from the integral values of the GMP H8 signals at $\delta = 8.05$ (H8, free) and $\delta = 8.72$ (H8*, platinated). The ratio of the integral values of H1′ at $\delta = 5.87$ and of H1′* at $\delta = 5.96$ was constant as well.

Table 1. NMR Data for the complexation of DAB(PA-tPt-Cl)$_4$ with GMP

| | δH8 | δH1′ | $J_{1'-2'}$ (Hz) | $\delta^{195}$Pt |
|---|---|---|---|---|
| GMP | 8.05 | 5.87 | 5.7 (d) | – |
| **DAB(PA-tPt-GMP)$_4$** | 8.72 | 5.96 | 3.6 (d) | −2560 |
| **DAB(PA-tPt-Cl)$_4$** | – | – | – | −2425 |

The values of H8* and H1′* are indicative of platination at N7. Also, the decrease in the coupling constant $J_{1'-2'}$ agrees with that observed before in the reaction of dinuclear 1,1/t,t compounds with GMP.[17] The $^{195}$Pt value of the GMP adduct lies near the values for dinuclear compounds as well. Apparently all four arms of the tetranuclear compound are available for binding to DNA. The binding of each separate arm is comparable to that of the ends of dinuclear 1,1/t,t compounds. However, the actual number of "arms" binding to DNA may be smaller in cells due to partial inactivation by binding to other cellular targets like GSH. This illustrates a feature of tetrafunctional compounds that might be advantageous compared to bifunctional compounds like cisplatin or the 1,1/t,t compounds. The tetrafunctional compound is still able to form crosslinks with DNA even after two of its functional groups are deactivated.

## Biological Activity

Cisplatin and the tetranuclear compound DAB(PA-tPt-Cl)$_4$ were tested on L1210/0 mouse leukemia cells, and on the cisplatin resistant L1210/2 cell line. These cell lines are described in literature.[19][20] The drug concentrations at 50% growth inhibition ($IC_{50}$) are presented in Table 2. As expected, cisplatin was found to be cytotoxic against the L1210/0 cells, and less cytotoxic in the resistant lines. The found $IC_{50}$ values are comparable to literature.[19][20] The resistance factor (rf, $IC_{50}$ for the resistant cell line divided by the $IC_{50}$ for the sensitive cells) was found to be 3.1. In contrast, the tetranuclear compound appeared to be equally toxic in L1210/0 and L1210/2 (rf = 0.8). However, the cytotoxicity of DAB(PA-tPt-Cl)$_4$ was low compared to cisplatin in both cell lines.

Table 2. $IC_{50}$ values obtained in cisplatin-sensitive L1010/0 mouse leukemia cells (A) and the resistant L1210/2 cell line (B). Resistance factors (*rf*) for cisplatin and DAB(PA)$_4$ were calculated (*rf* = B/A)

| | $IC_{50}$ (μM) L1210/0 (A) | Resistance factor L1210/2 (B) | *rf* = B/A |
|---|---|---|---|
| DAB(PA-tPt-Cl)$_4$ | 12.4 | 9.3 | 0.8 |
| cisplatin | 1.5 | 4.6 | 3.1 |

DAB(PA-tPt-Cl)$_4$ was also tested in 7 human tumor cell lines. $IC_{50}$ values are shown in Table 3. The tetranuclear compound showed a low cytotoxicity in all cell lines, which is in accordance with the results in L1210 mouse leukemia. In some cell lines, the $IC_{50}$ value could not be determined because it was out of the used concentration range. In the other cases, the $IC_{50}$ value of DAB(PA-tPt-Cl)$_4$ was found 13−20 times higher than that of cisplatin.

## Conclusion

The results in this paper show that it is possible to functionalize the four amino endgroups of the first generation of a poly(propyleneimine) dendrimer DAB(PA)$_4$ selectively with *trans*-diamminechloroplatinum moieties in high yield. The tetranuclear platinum compound DAB(PA-tPt-Cl)$_4$ is able to bind four molecules of GMP at their N7 positions. The arms of the tetranuclear compound are capable of binding GMP in a way similar to the ends of dinuclear 1,1/t,t compounds. The compound shows low cytotoxicity towards L1210/0 and L1210/2 mouse leukemia cell lines and seven human tumor cell lines. The low cytotoxicity may as well be caused by transport problems across the cell membranes the compound has to pass to reach DNA. The high charge (+6 at neutral or acidic pH) in combination with the branched structure may cause these problems. The high charge makes diffusion through the apolar membrane un-

Table 3. $IC_{50}$ values determined in seven human tumor cell lines using the microculture sulforhodamine B (SRB) test

| | $IC_{50}$ (μM) MCF7 | EVSA-T | WIDR | IGROV | M19 | A498 | H226 |
|---|---|---|---|---|---|---|---|
| **DAB(PA-tPt-Cl)$_4$** | 30.9 | 26.7 | > 40 | 8.9 | 26.2 | > 40 | > 40 |
| **cisplatin** | 2.3 | 1.4 | 3.2 | 0.6 | 1.9 | 7.5 | 10.9 |

likely. However, the efficient uptake of the trinuclear compound BBR 3464 (with a charge of +4) in L1210 cells[15] shows that a high charge does not necessarily hamper the influx of a compound. In literature it has been suggested that charged linear dinuclear compounds may enter the cell through the same gates and channels as the structurally related polyamines spermine and spermidine.[1] Such gates may be unable to accommodate transport of a compound with a branched structure such as DAB(PA-tPt-Cl)$_4$.

In order to establish a structure-activity relationship, we are currently preparing a family of di- and polynuclear platinum compounds. It would be interesting to study their cellular uptake and cytotoxicity, related to their charge and structure. This should improve our insight into the mechanism of action of this new class of platinum compounds.

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## Experimental Section

**General Remarks:** The complex *trans*-[$PtCl_2(NH_3)_2$] was prepared according to literature procedures[20] from $K_2PtCl_4$. $K_2PtCl_4$ was obtained from Johnson & Matthey (Reading, UK). Other chemicals were obtained from Aldrich and were used without further purification.

Titrations were carried out in $D_2O$. The pH was adjusted by adding trace amounts of 1 M DCl and NaOD to the NMR solutions. pH Measurements were carried out with a Radiometer PHM 80 pH meter, using a Hamilton combination glass electrode. The pH was corrected for the deuterium isotope effect by adding 0.4 units to the display readout. − NMR spectra were taken on a Bruker DPX 300 spectrometer with a 5-mm multi-nucleus probe. Temperature was kept constant by a variable temperature unit. $^1$H NMR spectra were determined with respect to external TMS. $^{195}$Pt spectra were calibrated by using $K_2PtCl_4$ as an external reference at δ = −1614.

### Syntheses

**[DAB(PA-*trans*-Pt($NH_3$)$_2$Cl]$Cl_4$** (DAB(PA-tPt-Cl)$_4$): This synthesis was based on literature procedures for the preparation of dinuclear 1,1/t,t compounds.[16] *trans*-$PtCl_2(NH_3)_2$ (0.70 g, 2.3 mmol) was activated by treatment with $AgNO_3$ (0.30 g, 2.3 mmol) in 13 mL of DMF. After stirring overnight in the dark, AgCl was removed by filtration. A solution of the ligand DAB(PA)$_4$ (0.16 g, 0.5 mmol) in 1 mL of DMF was added dropwise and the reaction mixture was stirred at 40°C in the dark. After 16 hours the reaction was quenched with a tenfold excess of LiCl. DMF was removed in vacuo, and the remaining yellow gel was treated with small portions of warm EtOH to remove LiCl. Unreacted *trans*-$PtCl_2(NH_3)_2$ was removed by adding a minimum amount of cold water, after which the remaining yellow solid was filtered off. Lyophilization of the solution yields an off-white solid (564 mg, 0.37 mmol, yield 90%). In some batches, small amounts of a brown sticky material developed after standing at room temperature for several weeks. This problem could be circumvented by storage at −20°C. More pure product has been obtained by repeated washing of the crude material with MeOH and warm EtOH. Mixing of the remaining solid with water, followed by filtration of the solution and lyophilization results in a white powder (0.13 g, 0.09 mmol, yield 17%). Analysis: $^1$H NMR ($D_2O$, pD = 12) δ (ppm) = 2.67 (t ($J$ = 7.2 Hz), 8 H, e), 2.60−2.45 (m, 12 H, b+c), 1.88 (m, 8 H, d), 1.46 (s, 4 H, a); $^{195}$Pt NMR ($D_2O$): δ (ppm) = −2425; calculated for $C_{16}H_{64}N_{14}Cl_8Pt_4$ (1516.72): C 12.67, H 4.25, N 12.93; found C 12.92, H 4.29, N 12.04. Although the nitrogen analysis is slightly lower (7%) than calculated, only one signal was observed in $^{195}$Pt NMR. Therefore it is concluded that the impurity is unlikely to be a platinum compound interfering in the cytotoxicity tests.

### Cytotoxic Studies

**Cytoxicity assays in L1210 cell lines were based on literature procedures.**[19][20] The cell lines L1210/0 and L1210/2 were cultured in McCoy's 5a medium supplemented with 10% fetal calf serum(Gibco, Paisley, Scotland), penicillin (100 units/ml: Duchefa, Netherlands) and streptomycin (100μg/ml: Duchefa, Netherlands). During growth, the cells grew partly in suspension and partly weakly adherent to the flasks. The cytotoxicity of cisplatin and DAB(PA-tPt-Cl)$_4$ were determined by measuring the inhibition of cell growth (cell growth assay, CGA). For the CGA, cells ($5 \times 10^4$/mL) were pre-cultured in 24 multi-well plates for 24 h at 37°C in an incubator with 5% $CO_2$ and subsequently treated with at least three different concentrations of cisplatin (0−16 μM) or DAB(PA-tPt-Cl)$_4$ (0−66 μM) for 3 days. Cell numbers were determined with a counting chamber. Each platinum concentration was tested in at least two independent experiments.

In vitro cytotoxicity assays in human tumor cell lines were performed at the Dr. Daniel den Hoed Kliniek (Rotterdam Cancer Institute), Department of Medical Oncology (Rotterdam, the Netherlands). The seven well-characterized cell lines used were MCF7 and EVSA-T (breast cancer), WIDR (colon cancer), IGROV (ovarian cancer), M19 (melanoma), A498 (renal cancer) and H226 (nonsmall cell lung cancer). $ID_{50}$ values were determined using the microculture sulforhodamine B test (SRB).[22]

[1] T. W. Hambley, *Coord. Chem. Rev.* **1997**, *166*, 181−223.
[2] M. J. Bloemink, J. Reedijk, *Metal Ions Biol. Syst.* **1996**, *32*, 641−685.
[3] J. Reedijk, *Chem. Commun.* **1996**, 801−806.
[4] S. G. Chaney, A. Sancar, *J. Nat. Cancer Inst.* **1996**, *88(19)*, 1346−1360.
[5] G. Chu, *J. Biol. Chem.* **1994**, *269(2)*, 787−790.
[6] D. P. Gately, S. B. Howell, *Br. J. Cancer* **1993**, *67*, 1171.
[7] N. Farrell, Y. Qu, L. Feng, B. Van Houten, *Biochemistry* **1990**, *29*, 9522.
[8] N. Farrell, *Cancer Invest.* **1993**, *11(5)*, 578−589.
[9] D. Yang, S. S. G. E. van Boom, J. Reedijk, J. H. van Boom, N. Farrell, A. H.-J. Wang, *Nature Struct. Biol.* **1995**, *2(7)*, 577−585.
[10] A. M. Fichtinger-Schepman, J. L. van der Veer, J. H. den Hartog, P. H. Lohman, J. Reedijk, *Biochemistry* **1985**, *24*, 707−713.
[11] A. Eastman, *Biochemistry* **1986**, *25*, 3912−3915.
[12] Y. Qu, N. Farrell, *J. Inorg. Biochem.* **1990**, *40*, 255.
[13] H. Rauter, R. Di Domenico, E. Menta, A. Oliva, Y. Qu, N. Farrell, *Inorg. Chem.* **1997**, *36*, 3919−3927.
[14] N. Farrell, E. Menta, M. Valsecchi, R. Di Domenico, G. Da Re, C. Manzotti, G. Pezzoni, F. C. Giuliani, S. Spinelli, *J. Inorg. Biochem.* **1997**, *67*, 173 (abstract).
[15] P. Di Blasi, A. Bernareggi, G. Beggiolin, L. Piazzoni, E. Menta, A. L. Formento, *Anticancer Res.* **1998**, *18*, 3113−3118.
[16] Y. Qu, N. Farrell, *Inorg. Chem.* **1992**, *31*, 930−932.
[17] Y. Qu, N. Farrell, *J. Am. Chem. Soc.* **1991**, *113*, 4851−4857.
[18] G. J. M. Koper, M. H. P. van Genderen, C. Elissen-Román, M. W. P. L. Baars, E. W. Meijer, M. Borkovec, *J. Am. Chem. Soc.* **1997**, *119*, 6512−6521.
[19] F. A. Blommaert, B. G. J. Floot, H. C. M. van Dijk-Knijnenburg, F. Berends, R. A. Baan, J. H. Schornagel, L. den Engelse, A. M. J. Fichtinger-Schepman, *Chemico-Biol. Interactions* **1998**, *108*, 209−225.
[20] A. Eastman, N. Schulte, *Biochemistry* **1988**, *27*, 275−280.
[21] G. B. Kaufman, D. O. Cowan, *Inorg. Synth.* **1963**, *7*, 239.
[22] Y. P. Kepers, G. J. Peters, J. Van Ark-Otte, B. Winograd, H. M. Pinedo, *Eur. J. Cancer* **1991**, *27*, 897−900.

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