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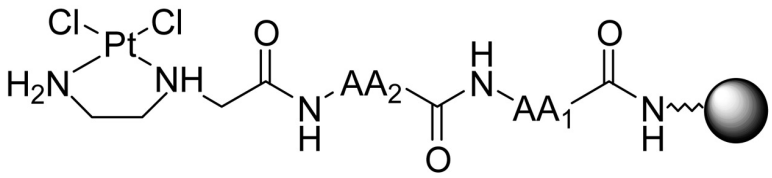


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# Automated Parallel Solid-Phase Synthesis and Anticancer Screening of a Library of Peptide-Tethered Platinum(II) Complexes

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The automated parallel solid-phase synthesis of a 36-member library of peptide-tethered platinum(II) complexes is described. The identity and quality of each product were confirmed by mass spectrometry and $^1$H NMR. Subsequently, each compound was screened for in vitro anticancer activity by treating the A2780 (human ovarian carcinoma) cell line with two concentrations of the drugs (100 and 10 $\mu$M) in quadruplicate. The reduction of cell proliferation induced by the drugs at these concentrations was determined with the MTT colorimetric assay (MTT = 3-(4′,5′-dimethylthiazol-2′-yl)-2,5-diphenyltetrazolium bromide) and compared to cisplatin. Even though no very active library members could be identified, five apparently most active (**8**{*1*}, **8**{*4*}, **8**{*10*}, **8**{*13*}, and **8**{*24*}) and two inactive complexes (**8**{*33*} and **8**{*34*}) were purified using gel permeation chromatography and fully characterized by NMR spectroscopy ($^1$H, $^{195}$Pt) and MS. The $IC_{50}$ values of these complexes and cisplatin in A2780 cells were subsequently determined using the MTT assay in a conventional manner. All seven complexes have an $IC_{50}$ above 100 $\mu$M, confirming the results generated by the assay at 100 and 10 $\mu$M of the crude reaction products.

## Introduction

In the last few decades, a plethora of platinum complexes have been synthesized to identify novel compounds with improved properties compared to the parent drugs (e.g., cisplatin and carboplatin) and to overcome cisplatin-related resistance in anticancer chemotherapy.[1] Unfortunately, only a few successful antitumor candidates have emerged so far. The use of combinatorial chemistry complemented by high-throughput screening is now a well-established protocol in drug discovery. A similar approach for inorganic compounds, with the exception of solid-state materials,[2] has been developed in only a few laboratories and has mainly dealt with catalyst design and enzyme mimics[2,3] and dynamic combinatorial libraries.[4] Interestingly, Lippard demonstrated that the platinum drug discovery process can be significantly accelerated by the preparation and mechanism-based screening of solution-phase platinum drug libraries.[5]

Solid-phase chemistry has been shown to be ideal for the preparation of libraries of potential therapeutic drugs, and the advantages involved are likely to pertain to platinum drugs. In this respect, we recently reported the convenient solid-phase synthesis of a dichloroplatinum(II) tripeptide complex (compound **1** in Figure 1).[6] The used solid-phase platination methodology is well-suited for the combinatorial or parallel synthesis and screening of a wide variety of dichloroplatinum(II) peptide complexes. The modular nature of complex **1** allows for the facile introduction of a high degree of diversity by varying the amino acids in the glycine-tethered dipeptide. Furthermore, as amino acid residues,[7] peptides,[3c,8] and polyamides[9] have been successfully employed as site-specific DNA-interacting elements conjugated to metal complexes, the use of peptides conjugated to a dichloroplatinum moiety could lead to specific and possibly favorable additional interactions with the platinated DNA.

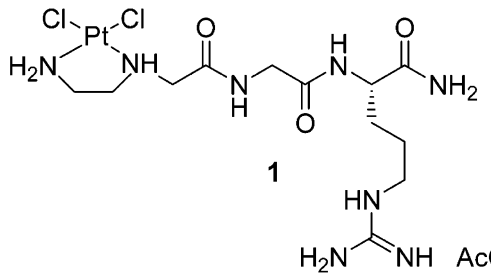


**Figure 1.** Dichloroplatinum(II) tripeptide complex **1**, synthesized on the solid support.

To initiate this work and to examine the scope and generality of the solid-phase platination approach, we set out to prepare a six by six array of individual dichloroplatinum peptide analogues of—and including—**1**, using a diverse set of six naturally occurring amino acids and an automated synthesizer. Evidently, a fast assay is required to screen a library of anticancer platinum complexes for the identification of new lead structures and elucidation of structure−activity relationships (SAR). Anticancer drugs are often developed using cell cytotoxicity as the primary screening method. Unfortunately, only small numbers of compounds can be conveniently screened in this way as it involves incubating cells in a range of concentrations (e.g., six) of a drug in quadruplicate. However, this technique has been adapted to

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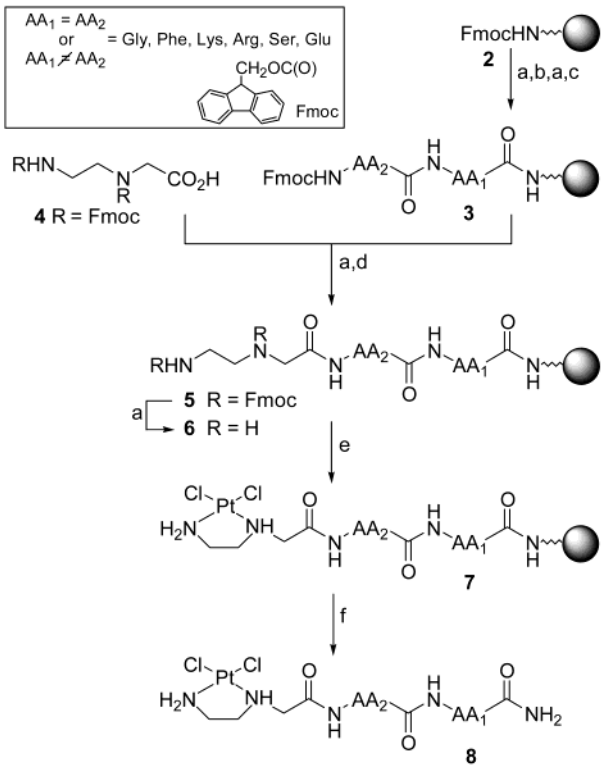
**Scheme 1.** Solid-Phase Synthesis of Library **8**[a]




[a] Reagents and conditions: (a) piperidine; (b) $AA_1$, BOP, HOBt, DiPEA, NMP; (c) $AA_2$, BOP, HOBt, DiPEA, NMP; (d) BOP, HOBt, DiPEA, NMP; (e) $K_2PtCl_4$ (5 equiv, 0.05 M), DMF/$H_2O$ (9/1, v/v), 48 h in the dark; (f) TFA/$H_2O$ (95/5, v/v), 1 h.

facilitate the assessment of larger numbers of compounds by testing drugs at a single concentration and determining whether the $IC_{50}$ is below or above this arbitrary concentration.[10] One of the most common and convenient ways of determining in vitro cell cytotoxicity uses the MTT colorimetric assay (MTT = 3-(4′,5′-dimethylthiazol-2′-yl)-2,5-diphenyltetrazolium bromide),[11] which allows quantitative spectrophotometric evaluation of cell proliferation in response to external factors, like drug treatment. This is based on the reduction of the yellow tetrazolium component (MTT) into an insoluble purple-colored formazan product by the mitochondria of viable cells. In this work, we present the first automated solid-phase preparation of a library of platinum complexes, complemented by subsequently screening the products at two concentrations, using the MTT assay.

## Results and Discussion

The general structure of the target library **8** is shown in Scheme 1 and encompasses a dipeptide tethered to an ethylenediamine moiety, which, in turn, serves as a platinum chelating ligand. The route of synthesis comprises the assembly of the immobilized and functionalized tripeptide derivative **5**, platination of the ethylenediamine moiety in **6**, and subsequent deprotection and release from the solid support. As constituents of the appended dipeptide in **8**, six amino acids were selected to encompass a wide range of substituent types: glycine, phenylalanine, lysine,[12] arginine, serine, and glutamate. Incorporating these amino acids in both positions of the appended dipeptide should result in a final library of 36 compounds (Table 1).

**Table 1.** Composition of Library **8**

| complex | $AA_1$ | $AA_2$ |
|---|---|---|
| *1* | Gly | Gly |
| *2* | Gly | Phe |
| *3* | Gly | Lys |
| *4* | Gly | Arg |
| *5* | Gly | Ser |
| *6* | Gly | Glu |
| *7* | Phe | Gly |
| *8* | Phe | Phe |
| *9* | Phe | Lys |
| *10* | Phe | Arg |
| *11* | Phe | Ser |
| *12* | Phe | Glu |
| *13* | Lys | Gly |
| *14* | Lys | Phe |
| *15* | Lys | Lys |
| *16* | Lys | Arg |
| *17* | Lys | Ser |
| *18* | Lys | Glu |
| *19* | Arg | Gly |
| *20* | Arg | Phe |
| *21* | Arg | Lys |
| *22* | Arg | Arg |
| *23* | Arg | Ser |
| *24* | Arg | Glu |
| *25* | Ser | Gly |
| *26* | Ser | Phe |
| *27* | Ser | Lys |
| *28* | Ser | Arg |
| *29* | Ser | Ser |
| *30* | Ser | Glu |
| *31* | Glu | Gly |
| *32* | Glu | Phe |
| *33* | Glu | Lys |
| *34* | Glu | Arg |
| *35* | Glu | Ser |
| *36* | Glu | Glu |

The parallel synthesis of the dichloroplatinum−peptide array was performed with an automated synthesizer on a 50 $\mu$mol scale. Two rounds of stepwise elongation of the Fmoc-protected Rink amide resin (**2**) with the six commercially available protected amino acids, following a standard Fmoc protocol,[13] resulted in the immobilized dipeptides **3**. Condensation of the latter with the platinum-binding unit **4** afforded immobilized trimers **5**.[14] Removal of both Fmoc-protecting groups in **5** led to tripeptides **6**. Treatment with excess $K_2PtCl_4$ (5 equiv, 0.05 M) in DMF/water (9/1) for 48 h afforded immobilized dichloroplatinum complexes **7**. Concomitant cleavage of the amino acid-protecting groups and release from the solid support with TFA/water (95/5) for 1 h was followed by precipitation of the TFA solution with cold diethyl ether, yielding 36 beige or yellow solids (**8**). All 36 library members targeted for production were successfully synthesized, as evidenced by $^1$H NMR and mass spectrometry (MS). Conversion of the immobilized ligands **6** into the immobilized platinum complexes **7** ranged from 82 to 92%, as gauged by $^1$H NMR of the crude complexes **8**. These values are slightly lower than the 96% previously found for **1**,[15] which is probably due to the difference in mixing properties of the reactors in the synthesizer as compared to a syringe or a flask. In all cases, the expected dichloroplatinum complex, as well as a small amount of unreacted free ligand, could be observed in MS, confirming the successful automated synthesis of the ligand, as well as the corresponding platinum complex.

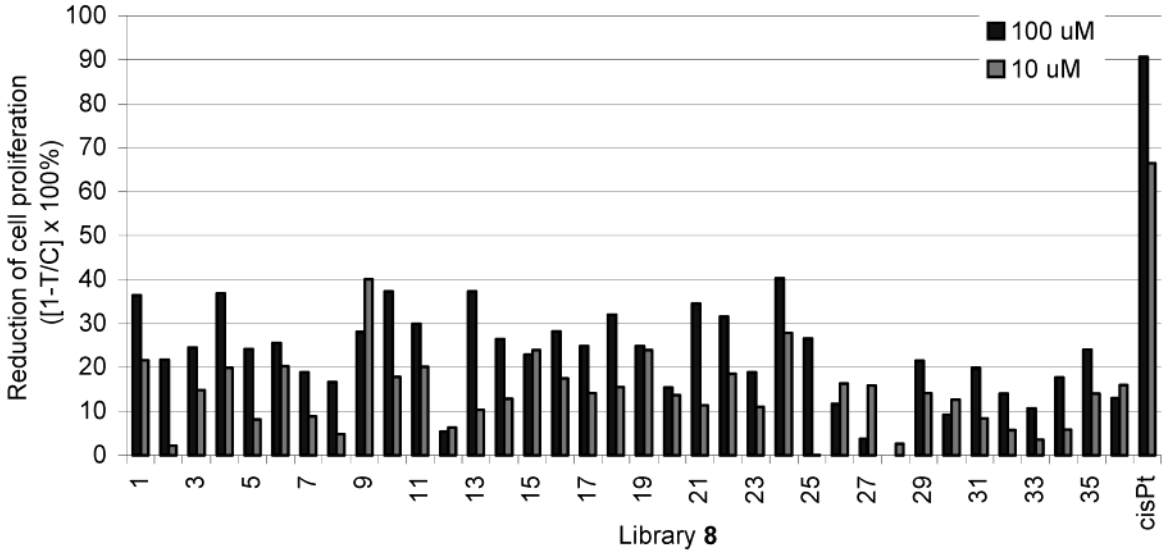



**Figure 2.** Reduction of cell proliferation in the A2780 cell line by library **8** and cisplatin at 10 and 100 $\mu$M.

Individual members of the array were subsequently screened for in vitro anticancer activity by determining the cytotoxicity in the A2780 cell line at concentrations of 10 and 100 $\mu$M. To this end, 96-well microplates with A2780 cells were incubated for 3 days with the two concentrations of the compounds in quadruplicate. Cell viability was determined with the MTT colorimetric assay, and the results are expressed as % reduction of cell proliferation, obtained by dividing the optical density values (OD) of the treated groups (T) by the OD of the controls (C) ([1 − T/C × 100%, Figure 2).[16] Inspection of the reduction of cell proliferation induced by the library indicates that all compounds have an $IC_{50}$ of more than 100 $\mu$M, because the most active complexes exhibit a cell growth reduction of around 40% at this concentration. Despite the apparent inactivity of the library, a good correlation is found between the values for 100 and 10 $\mu$M. Cisplatin causes a reduction of cell proliferation of 91% at 100 $\mu$M and 66% at 10 $\mu$M, demonstrating that the conditions and dilutions used in the experiments were appropriate.

To ascertain that testing crude solid-phase reaction products at two concentrations provides a reliable indication of their $IC_{50}$ value, five apparently most active (**8**{*1*}, **8**{*4*}, **8**{*10*}, **8**{*13*}, and **8**{*24*}) and two inactive complexes (**8**{*33*} and **8**{*34*}) were purified using gel permeation chromatography (HW-40, 0.01 M HCl in water/methanol (1/1)) and their identity was fully confirmed by NMR spectroscopy ($^1$H, $^{195}$Pt) and MS. Subsequently, the $IC_{50}$ values of these complexes and cisplatin in the A2780 cell line were determined using the MTT assay in a conventional manner. Except for complex **8**{*1*}, displaying an $IC_{50}$ of 149 $\mu$M, the $IC_{50}$ values of the complexes could not be determined, because the values are well above 100 $\mu$M. Cisplatin exhibited an $IC_{50}$ of 3 $\mu$M, which correlates nicely with the activity found in the assay. Although no very active new complexes were found, the activity of the crude complexes and cisplatin measured in the assay was found to correlate well with the (lack of) activity found for the purified and characterized compounds and cisplatin measured in the conventional $IC_{50}$ determination. This demonstrates that the MTT colorimetric assay, when applied at two suitable concentrations, is an effective system for screening crude platinum drug libraries generated through solid-phase chemistry.

## Conclusion

The work described above serves to illustrate the opportunities of the application of automated parallel solid-phase synthesis and screening in platinum drug research. It has been firmly established that our previously published solid-phase platination methodology is robust and widely applicable. Using this technique, a library of platinum complexes of high quality has been generated via solid-phase synthesis for the first time and has also been conveniently and reliably screened for cytotoxicity with the MTT assay, without the need for purification. Although these platinum peptide complexes show no promise as cytotoxic agents, this work clearly demonstrates the utility of automated solid-phase synthesis of platinum drugs, complemented by the MTT in vitro screening method, as the whole process takes considerably less time than conventional synthesis and testing would have required. The solid-phase platination technique is likely to be amendable toward the preparation of larger libraries via a split-mix protocol, as well as the preparation of platinum drug libraries based on small nonoligomeric molecules, generated by solid-phase organic chemistry.

## Experimental Section

**General.** All solvents and reagents used in the automated peptide synthesis were of peptide synthesis grade and were purchased from Biosolve. Amino acids and the Rink Amide MBHA resin were purchased from NovaBiochem. Compound **4** was synthesized according to our previously published procedure.[6] Gel permeation chromatography was executed on an HW-40 column (26 mm × 600 mm) at 1.5 mL/min. Mass spectra were recorded on a PE SCIEX API 165 instrument. NMR spectra were taken on a Bruker DPX 300 spectrometer with a 5 mm multinucleus probe. Temperature was kept constant at 295 K by a variable temperature unit. $^1$H NMR was measured using TMS as an external

reference at $\delta = 0$ ppm. $^{195}$Pt NMR spectra were calibrated with respect to external $K_2PtCl_4$ at $\delta = -1614$ ppm.

**Automated Solid-Phase Synthesis of Library 8.** Solid-phase synthesis of **8** was carried out in parallel on a LaMOSS (Labotec Modular Organic Synthesis System) robot synthesizer on 50 $\mu$mol scale. Stepwise elongation of the Rink amide resin employing a standard *Fastmoc* peptide synthesis protocol gave immobilized tripeptides **6**. The amino acids used were Fmoc−Gly−OH, Fmoc−Phe−OH, Fmoc−Lys(Boc)−OH, Fmoc−Arg(Pbf)−OH, Fmoc−Ser(*t*Bu)−OH, and Fmoc−Glu(*t*Bu)−OH. The first coupling step was repeated to ensure a high loading. After assembly of **6**, the peptide-resins were washed with MeOH/$CH_2Cl_2$ and $CH_2Cl_2$ and dried to the air. Subsequently, the reactors were loaded with $K_2PtCl_4$ (5 equiv, 0.05 M) in DMF/$H_2O$ (9/1, v/v) and covered with aluminum foil. The peptide-resins were suspended by a stream of nitrogen every 5 min for 2 days. Afterward, the reaction mixtures were filtered and the resins were washed with DMF/$H_2O$, $H_2O$/MeOH, MeOH/$CH_2Cl_2$, and $CH_2Cl_2$. Cleavage of the protecting groups and the solid support was effected by treatment of **7** with TFA/$H_2O$ (95/5, v/v) for 1 h after which the reaction mixtures were filtered, concentrated, and precipitated with cold diethyl ether. The resulting solids were washed with diethyl ether twice and dried to the air.

After the initial cytotoxicity screening, complexes **8**{*1*}, **8**{*4*}, **8**{*10*}, **8**{*13*}, **8**{*24*}, **8**{*33*}, and **8**{*34*} were purified by gel-permeation chromatography (HW-40, 0.01 M HCl in $H_2O$/MeOH). The yields of the purified complexes are based on the initial loading of the Rink amide resin.

***N*-(2-Aminoethyl)glycine-glycine-glycine-$NH_2$-dichloroplatinum 8{*1*}.** Yellow powder, 58% yield. $^1$H NMR ($D_2O$): $\delta$ 4.06 (m, 1H; $CH_2$), 4.03 (AB, 2H; αGly), 3.95 (s, 2H; αGly), 3.77 (m, 1H; $CH_2$), 3.05−2.86 (br m, 1H; $CH_2CH_2$), 2.82−2.52 (br m, 3H; $CH_2CH_2$). $^{195}$Pt NMR ($D_2O$): $\delta$ −2376. ESI-MS: *m*/*z* 496 $[M + H]^+$.

***N*-(2-Aminoethyl)glycine-arginine-glycine-$NH_2$-dichloroplatinum HCl 8{*4*}.** Yellow powder, 63% yield. $^1$H NMR ($D_2O$): $\delta$ 4.34 (m, 1H; αArg), 3.94 (m, 1H; $CH_2$), 3.91 (s, 2H; αGly), 3.66 (m, 1H; $CH_2$), 3.19 (m, 2H; $\delta$Arg), 2.81 (br m, 1H; $CH_2CH_2$), 2.75−2.50 (br m, 3H; $CH_2CH_2$), 1.94−1.58 (m, 4H; $\beta$Arg, $\gamma$Arg). $^{195}$Pt NMR ($D_2O$): $\delta$ −2372. ESI-MS: *m*/*z* 595 $[M + H]^+$.

***N*-(2-Aminoethyl)glycine-arginine-phenylalanine-$NH_2$-dichloroplatinum HCl 8{*10*}.** Yellow powder, 47% yield. $^1$H NMR ($D_2O$): $\delta$ 7.34 (m, 5H; Phe arom.), 4.67 (m, 1H; αPhe), 4.25 (m, 1H; αArg), 3.91 (m, 1H; $CH_2$), 3.74 (m, 1H; $CH_2$), 3.25 (ABX, 1H; $\beta$Phe), 3.07 (m, 3H; $\delta$Arg, $\beta$Phe), 2.90−2.55 (br m, 4H; $CH_2CH_2$), 1.61 (m, 2H; $\beta$Arg), 1.37 (m, 2H; $\gamma$Arg). $^{195}$Pt NMR ($D_2O$): $\delta$ −2371. ESI-MS: *m*/*z* 685 $[M + H]^+$.

***N*-(2-Aminoethyl)glycine-glycine-lysine-$NH_2$-dichloroplatinum HCl 8{*13*}.** Yellow powder, 46% yield. $^1$H NMR ($D_2O$): $\delta$ 4.32 (m, 1H; αLys), 4.06 (m, 1H; $CH_2$), 4.02 (s, 2H; αGly), 3.77 (m, 1H; $CH_2$), 3.03 (t, $J = 7.2$ Hz, 2H; $\epsilon$Lys), 2.91 (br m, 1H; $CH_2CH_2$), 2.70 (br m, 3H; $CH_2CH_2$), 1.85 (m, 2H; $\beta$Lys), 1.71 (m, 2H; $\delta$Lys), 1.48 (m, 2H; $\gamma$Lys). $^{195}$Pt NMR ($D_2O$): $\delta$ −2372. ESI-MS: *m*/*z* 567 $[M + H]^+$.

***N*-(2-Aminoethyl)glycine-glutamate-arginine-$NH_2$-dichloroplatinum HCl 8{*24*}.** Yellow powder, 21% yield. $^1$H NMR ($D_2O$): $\delta$ 4.48−4.28 (m, 2H; αArg, αGlu), 4.09−3.67 (m, 2H; $CH_2$), 3.23 (t, $J = 6.7$ Hz, 2H; $\delta$Arg), 3.05−2.55 (br m, 4H; $CH_2CH_2$), 2.51 (m, 2H; $\gamma$Glu), 2.14 (m, 1H; $\beta$Glu), 2.01 (m, 1H; $\beta$Glu), 1.86 (m, 2H; $\beta$Arg), 1.67 (m, 2H; $\gamma$Arg). $^{195}$Pt NMR ($D_2O$): $\delta$ −2375. ESI-MS: *m*/*z* 667 $[M + H]^+$.

***N*-(2-Aminoethyl)glycine-lysine-glutamate-$NH_2$-dichloroplatinum HCl 8{*33*}.** Yellow powder, 35% yield. $^1$H NMR ($D_2O$): $\delta$ 4.35 (m, 2H; αLys, αGlu), 4.00 (m, 1H; $CH_2$), 3.75 (m, 1H; $CH_2$), 3.01 (t, $J = 7.2$ Hz, 2H; $\epsilon$Lys), 2.88 (br m, 1H; $CH_2CH_2$), 2.69 (br m, 3H; $CH_2CH_2$), 2.51 (t, $J = 6.9$ Hz, 2H; $\gamma$Glu), 2.17 (m, 1H; $\beta$Glu), 2.02 (m, 1H; $\beta$Glu), 1.81 (m, 2H; $\beta$Lys), 1.71 (m, 2H; $\delta$Lys), 1.48 (m, 2H; $\gamma$Lys). $^{195}$Pt NMR ($D_2O$): $\delta$ −2372. ESI-MS: *m*/*z* 639 $[M + H]^+$.

***N*-(2-Aminoethyl)glycine-arginine-glutamate-$NH_2$-dichloroplatinum HCl 8{*34*}.** Yellow powder, 36% yield. $^1$H NMR ($D_2O$) $\delta$ 4.38 (m, 2H; αGlu, αArg), 4.01 (m, 1H; $CH_2$), 3.74 (m, 1H; $CH_2$), 3.23 (t, $J = 6.2$ Hz, 2H; $\delta$Arg), 2.87 (br m, 1H; $CH_2CH_2$), 2.68 (br m, 3H; $CH_2CH_2$), 2.51 (t, $J = 7.1$ Hz, 2H; $\gamma$Glu), 2.14 (m, 1H; $\beta$Glu), 2.03 (m, 1H; $\beta$Glu), 1.84 (m, 2H; $\beta$Arg), 1.69 (m, 2H; $\gamma$Arg). $^{195}$Pt NMR ($D_2O$): $\delta$ −2374. ESI-MS: *m*/*z* 667 $[M + H]^+$.

**In Vitro Cytotoxicity Studies. Cell Lines.** A2780 (human ovarian carcinoma) and A2780 cisplatin-resistant cell lines were cultured in complete medium consisting of DMEM (Gibco BRL, Invitrogen Corporation, Netherlands) supplemented with 10% bovine calf serum (Perbio Science, Belgium), 0.1% PenicillinG Sodium 1000x (Duchefa Biochemie BV, Netherlands), 0.1% Streptomycin 1000x (Duchefa Biochemie BV) and Glutammax 100x (Gibco BRL). The cells were passed twice a week, when on confluence, using diluted Trypsin (0.25×).

**Evaluation of the Reduction of Cell Proliferation**. For the evaluation of the reduction of proliferation, 2000 cells/100 $\mu$L complete medium/well were seeded in 96-multiwell flatbottom microtiter plates (Corning Costar). The plates were incubated at 37 °C, 5% $CO_2$ for 48 h prior to drug testing to allow cell adhesion. The tested concentrations for all compounds were 100 and 10 $\mu$M. Stock solutions (2 mM in water or in complete medium with 5% DMF) of all compounds were freshly prepared. The dilutions (1:10 and 1:100) were prepared in complete medium. Each concentration was tested in quadruplicate, adding 100 $\mu$L/well (containing cells grown in 100 $\mu$L complete medium). The control groups were treated with 100 $\mu$L of complete medium containing the same amount of water or DMF as used for treated groups (10% water for 100 $\mu$M or 1% for 10 $\mu$M compounds dissolved in water; 0.5% DMF for 100 $\mu$M or 0.05% for 10 $\mu$M compounds dissolved in complete medium with 5% DMF). The cells were incubated with the compounds for 72 h, and the evaluation of cell proliferation was performed by the MTT colorimetric assay.[11]

**MTT Colorimetric Assay.[11]** MTT solution (50 $\mu$L, 5 mg/mL in PBS, Sigma Chemical Co.) was added to each well and incubated for 2 h. The formed formazan crystals were dissolved with 100 $\mu$L of DMSO. OD was measured by

microplate reader (Bio Rad) at 590 nm. The reduction of cell proliferation was determined by reporting OD values of T with the OD of the appropriate C and expressed as $(1 - T/C) \times 100\%$.

**$IC_{50}$ Determination.** Stock solutions of selected compounds (2 mg/mL complete medium) were freshly prepared. The dilutions (6 step dilutions) were prepared in complete medium. The range of the concentrations used was 0.2−0.04−0.02−0.004−0.002−0.0004 mg/mL. Each concentration was tested in quadruplicate using 100 $\mu$L/well added to the 100 $\mu$L of complete medium. In the control group, only 100 $\mu$L of complete medium was added. The plates were incubated for 72 h, and the evaluation of cell proliferation was performed by the MTT colorimetric assay, as described above. $IC_{50}$ values were obtained by GraphPad Prism software, version 3.05, 2000.

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(12) A preliminary investigation demonstrated that it was unlikely that the library members containing a pendant lysine residue would exhibit an intramolecular coordination of the $\epsilon NH_2$ moiety to the platinum. Individually synthesized lysine-containing **8**{*13*} was dissolved in $D_2O$ at pH 7.2 (40 mM $KD_2PO_4$), and subjected to a series of $^{195}Pt$ NMR measurements over a period of 12 h. The only signal observed was the expected $PtN_2Cl_2$ chromophore at −2381 ppm, demonstrating that lysine could safely be included as a library component.
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(15) Compound **1** corresponds to library member **8**{*19*}.
(16) Library **8** showed no activity in the A2780 cisplatin resistant cell line in an identical assay (data not shown).

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