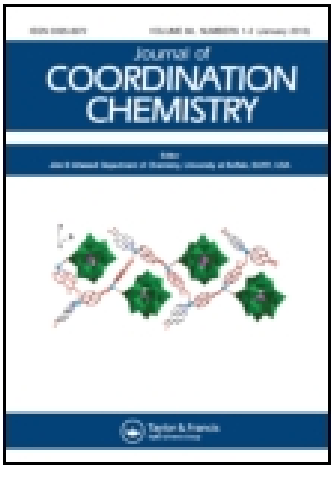

Journal of Coordination Chemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gcoo20

Preparation and in vitro cytotoxicity of oxaliplatin derivatives with chiral amino acid as the carrier group

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Accepted author version posted online: 17 Jul 2014.Published online: 11 Aug 2014.

To cite this article: Chuanzhu Gao, Tianshuai Wang, Ji Chen, Yan Zhang, Bo Yang & Shaohua Gou (2014) Preparation and in vitro cytotoxicity of oxaliplatin derivatives with chiral amino acid as the carrier group, Journal of Coordination Chemistry, 67:13, 2195-2203, DOI: 10.1080/00958972.2014.945922

To link to this article: http://dx.doi.org/10.1080/00958972.2014.945922

Journal of Coordination Chemistry, 2014
Vol. 67, No. 13, 2195–2203, http://dx.doi.org/10.1080/00958972.2014.945922

Preparation and *in vitro* cytotoxicity of oxaliplatin derivatives with chiral amino acid as the carrier group

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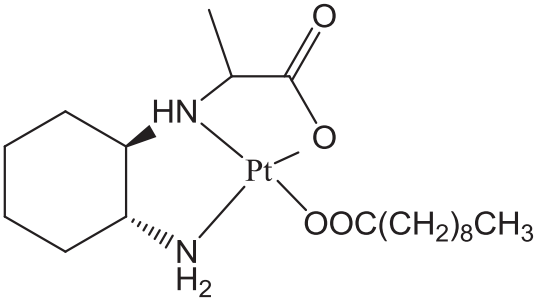
(*Received 5 November 2013; accepted 4 June 2014*)



P4

Novel platinum(II) compounds with a new chiral ligand were designed, prepared and biologically evaluated. Results indicated that compounds **P3** and **P4** showed better antitumor activity than carboplatin against two selected human cell lines.

Eight oxaliplatin derivatives with chiral amino acid, 2-{[(1R,2R)-2-aminocyclohexyl]amino}propanoic acid, as the carrier group, were designed, synthesized, and spectrally characterized by IR, ^{1}H NMR, MS spectra, and microanalyses. *In vitro* cytotoxicities against human HepG-2, MCF-7, A549, and HCT-116 cell lines were evaluated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazo-liumbromide assay. Results indicated that all compounds exhibited sensitivity to HepG-2 cell line, and among them, compounds **P3** and **P4** which have $CH_3(CH_2)_6COO^-$ and $CH_3(CH_2)_8COO^-$ as the leaving groups, respectively, gave better antitumor activity than carboplatin against HepG-2 and A549 cell lines.

Keywords: Chiral amino acid; Platinum(II) complexes; *In vitro* cytotoxicity

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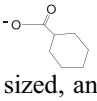
1. Introduction

Cisplatin is one of the most frequently used chemotherapeutics in the treatment of malignant tumors. However, clinical application of cisplatin is greatly limited by its side effects including nephrotoxicity and neurotoxicity [1], narrow range of activity, intrinsic and/or acquired resistance, and low aqueous solubility [2]. So far, tremendous efforts have been devoted to developing new platinum compounds with improved pharmacological properties and broader range of antitumor activity. This led to clinical development of cisplatin analogs such as carboplatin and oxaliplatin which have been widely used for treating ovarian cancers and colorectal tumors, respectively [3, 4].

Oxaliplatin, the first platinum-based drug owning a chiral moiety, 1R, 2R-diaminocyclohexane (DACH), as the carrier group, has became the first line agent in treating advanced colorectal cancer [5] and surpassed cisplatin as the biggest sale in this type of antitumor drug. However, dose-limiting toxicity of oxaliplain is still serious. It has been reported that 90% of patients treated with oxaliplatin suffered from acute neurotoxicity, while 10–15% of patients suffered from cumulative sensory [6]. Like other clinically available platinum anticancer drugs, such as carboplatin and lobaplatin, oxaliplatin cannot offer any substantial clinical advantages over the existing cisplatin [7].

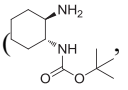
Amino acids are the elementary units for composing all kinds of proteins. Introducing amino acid into antitumor drugs could improve their target characteristics, relieve the toxicities to normal cells, enhance the curative effect for tumors and take advantage of the O/N groups as donors or acceptors for hydrogen bonds with DNA to further stabilize the DNA-drug adducts [8]. Pt–amino acid complexes showed potential applications as antitumor drugs such as DNA binding properties, HMG1 protein affinity for the platinated DNA and cytotoxicity against HeLa cells [9]. More recently, synthetic amino acids showed better qualities than natural amino acids in preparing Pt–amino acids with peptides incorporating non-natural amino acids showing increased metabolic stability and, in some cases, also enhanced biological activity [10, 11].

Our group have been studying new oxaliplatin derivatives that could improve their therapeutic effects and reduce their side effects [12–16]. Expecting interesting characteristics of Pt complexes of an unnatural amino acid, and in order to overcome the drawbacks of oxaliplatin, we envisioned that introducing suitable carboxylic acids to DACH may offer some new unnatural amino acids that could be taken as ligands to prepare Pt–amino acid complexes. In this paper, an unnatural amino acid, 2-{[(1R,2R)-2-aminocyclohexyl]amino} propanoic acid (HP) was prepared as the carrier group, and with CH_3COO^-, $ClCH_2COO^-$, $CH_3(CH_2)_6COO^-$, $CH_3(CH_2)_8COO^-$, $CH_3(CH_2)_{10}COO^-$, $CH_3(CH_2)_{12}COO^-$,  , and  as the leaving group, eight platinum(II) complexes (**P1–P8**) were designed, synthesized, and spectrally characterized by IR, ^{1}H NMR, and MS spectra. Their *in vitro* cytotoxicities against four human cancer cell lines were also reported.

2. Experimental

2.1. *Materials*

K_2PtCl_4 was purchased from a local chemical company. The optically pure (1R,2R) *trans*-cyclohexanediamine, DACH, was purchased from a Chinese chemical company,

Aladdin reagent. Mono-Boc protecting DACH ( , hereinafter **1**) was used as starting material and prepared according to the procedure reported [17].

All reagents and solvents were of analytical grade and used without purification. RPMI-1640 medium, trypsin, and fetal bovine serum were purchased from Gibco. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazo-liumbromide (MTT), benzyl penicillin, and streptomycin were from Sigma. Four different human carcinoma cell lines, HepG-2 (human hepatocellular carcinoma cell), MCF-7 (human breast cancer cell), A549 (human lung cancer cell), and HCT-116 (human colorectal cancer cell), were obtained from American Type Culture Collection.

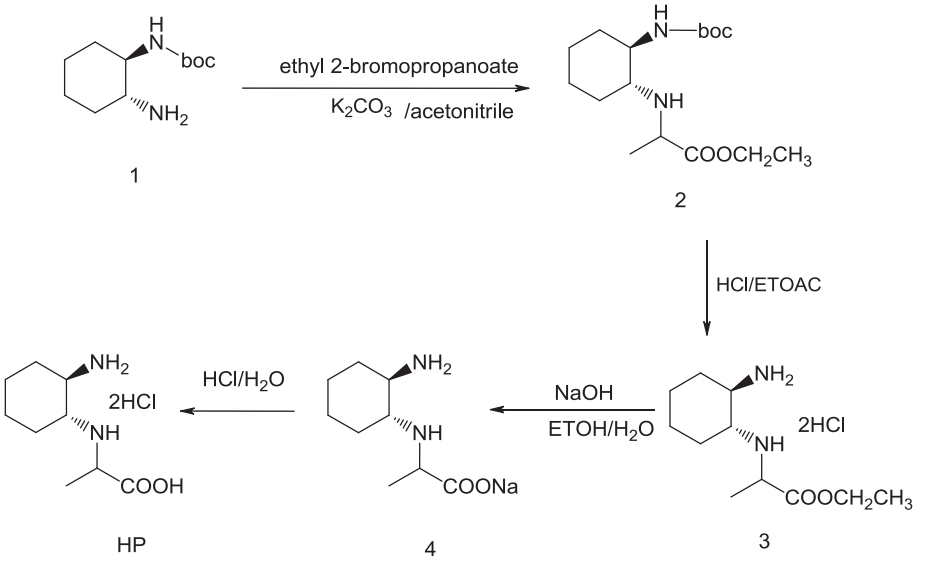
2.2. *Instrumentation and measurement*

Elemental analyses for C, H, and N were carried out with a Perkin-Elmer 1400C instrument. ESI-MS spectra were performed on a Finnigan MAT SSQ 710 (120–1000 am) apparatus and IR spectra were scanned with a Nicolet IR200 spectrophotometer from 4000 to 400 cm^{-1} in KBr pellets. ^1H NMR spectra were recorded on a Bruker DRX-500 Avance spectrometer at 500 MHz in D_2O using TMS as an internal reference. Specific rotations were tested on a Shenguang WZZ-2B instrument.

2.3. *Synthesis of compounds*

2.3.1. 2-{[(1R,2R)-2-aminocy-clohexyl]amino}propanoic acid (HP). 25.7 g mono-boc protected DACH (**1**) was dissolved in 500 mL acetonitrile, then 18.7 g K_2CO_3 and 21.7 g ethyl 2-bromopropanoate were added and refluxed for 6 h. Column chromatography (developer: petroleum ether/acetic ester/methanol = 5/1/1) gave intermediate **2**. Dissolving **2** in 200 mL methanol with stirring, excessive HCl/Methanol was added and then a white solid **3** precipitated. Intermediate **3** was hydrolyzed in aqueous NaOH solution and intermediate **4** was obtained, which was then neutralized by HCl/Methanol to obtain a faint yellow solid of HP; the yield was 38% (scheme 1). Anal. Calcd for $C_9H_{20}Cl_2N_2O_2$: C, 41.71; H, 7.78; N, 10.81. Found (%): C, 41.58; H, 7.80; N, 10.74. IR (KBr): 3343s(br), 2969s, 2928s, 2855s, 1583(sh)vs 1444vs, 1337m, 1302m, 1144m, 1074w, 976w, 916m, 880m, 867m, 768m, 598m, 436m. ^1H NMR (D_2O/TMS): δ 0.86 (d, 3H, CH_3, $J = 7.0$) 1.04–1.08 (m, 4H, $2CH_2$ of DACH), 1.54–1.87 (m, 4H, $2CH_2$ of DACH), 2.00–2.26 (d, 2H, 2*CH* of DACH), 3.19–3.21 (m, 1H, NH*CH*(CH_3)COO). ESI-MS *m/z*: $[M - H - 2HCl]^- = 185.6$ (100%).

2.3.2. Compound [PtRI]. To a stirred aqueous solution of KI (160 mM), K_2PtCl_4 (24 mM) in water (80 mL) was added. The solution was stirred at 25 °C for 30 min under a nitrogen atmosphere to get a black solution of K_2PtI_4. Then an aqueous solution (40 mL) of HP (24 mM) and NaOH (24 mM) were added dropwise under stirring in the dark at 25 °C. After 24 h, yellow precipitate was filtered, washed sequentially with water, ethanol, and ether, and then dried in vacuum as a yellow solid. Yield: 58%. Data for [PtRI]: Anal. Calcd for $C_9H_{17}IN_2O_2Pt$ (%): C, 21.31; H, 3.38; N, 5.52. Found (%): C, 21.22; H, 3.30; N, 5.54. IR (KBr, cm^{-1}): 3467m, 3171s, 3113s, 2929m, 2856m, 1648vs, 1447m, 1376m, 1337m, 1260m, 1195w, 1140w, 1103w, 1021w, 872w, 617w, 459w. ^1H NMR (D_2O/TMS, ppm):



Scheme 1. Synthetic scheme for HP.

δ 0.84 (s, 3H, CH_3), 1.08–1.91 (m, 8H, $4CH_2$ of DACH), 2.27–2.93 (d, 2H, *2CH* of DACH), 4.24 (s, 1H, NH*CH*(CH_3)COO). ESI-MS *m/z*: $[M + Na]^+ = 530.0$ (75%), $[M + K]^+ = 546$ (100%).

2.3.3. Compound P1. Four millimole [PtRI] was suspended in 250 mL pure water and a solution of 4 mM $AgNO_3$ in 80 mL pure water was added. After stirring under a nitrogen atmosphere in the dark for 24 h at 50 °C, the precipitate was filtered off. Filtrate was blended with 4 mM CH_3COONa and stirred at 55 °C for 24 h. The solution was concentrated to 3 mL and then cooled to 0 °C. A pale yellow powder was collected, washed with a small amount of chilled water and ethanol, and then dried at 60 °C in vacuum. Yield 23.6%. Anal. Calcd for $C_{11}H_{20}N_2O_4Pt$ (%): C, 30.07; H, 4.59; N, 6.38. Found (%): C, 30.10; H, 4.66; N, 6.56. IR (KBr, cm^{-1}): 3408m, 3100m, 2938m, 2862m, 1598s, 756m. ^1H NMR (D_2O, ppm): δ 0.98–3.03 (m, 16H, 10H of DACH and 3H of CH_3COO and 3H of NHCH(CH_3)COO), 4.26 (s, 1H, NH*CH*(CH_3)COO). ESI-MS *m/z:* $[M - H]^- = 438$ (100%).

The procedures for preparing **P2**–**P8** were similar to **P1**.

2.3.4. Compound P2. The leaving group of compound **P2** is $ClCH_2COO^-$, so the starting material was $ClCH_2COONa$. Yield 26.0%. Anal. Calcd for $C_{11}H_{19}ClN_2O_4Pt$ (%): C, 27.88; H, 4.04; N, 5.91. Found (%): C, 27.96; H, 4.07; N, 5.96. IR (KBr, cm^{-1}): 3411m, 3103m, 2936m, 2866m, 1596s, 751m. ^1H NMR (D_2O, ppm): δ 1.01–3.06 (m, 15H, 10H of DACH and 2H of ClCH_2COO and 3H of NHCH(CH_3)COO), 4.28 (s, 1H, NH*CH*(CH_3)COO). ESI-MS *m/z:* $[M - H]^- = 472$ (80%).

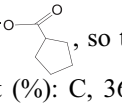
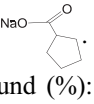
2.3.5. Compound P3. The leaving group of compound **P3** is $CH_3(CH_2)_6COO^-$, so the starting material was $CH_3(CH_2)_6COONa$. Yield 49.1%. Anal. Calcd for $C_{17}H_{32}N_2O_4Pt$ (%): C, 39.00; H, 6.16; N, 5.35. Found (%): C, 39.02; H, 6.22; N, 5.36. IR (KBr): 3479m,

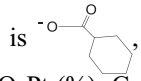
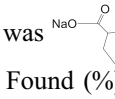
3250m, 3097m, 2922s, 2855m, 1577vs(sh), 1468m, 1388m, 1303m, 1153w, 1106m, 1039w, 873w, 623w, 459w. ^{1}H NMR (DMSO/TMS): δ 1.13–2.66 (m, 28H, 3H of NHCH (CH_3)COO and 10H of DACH and 15H of COO$(CH_2)_6CH_3$), 3.49–3.55 (m, 1H, NH*CH* (CH_3)COO), 5.30–6.19 (3H, NH and NH_2). ESI-MS *m/z*: $[M + H]^+ = 524.0$ (100%).

2.3.6. Compound P4. The leaving group of compound **P4** is $CH_3(CH_2)_8COO^-$, so the starting material was $CH_3(CH_2)_8COONa$. Yield 44.0%. Anal. Calcd for $C_{19}H_{36}N_2O_4Pt$ (%): C, 41.37; H, 6.58; N, 5.08. Found (%): C, 41.29; H, 6.62; N, 5.11. IR (KBr): 3478m, 3240m, 3097m, 2954s, 2850m, 1654s, 1559vs, 1447m, 1384m, 1107w, 835w, 722w, 696w. ^{1}H NMR (DMSO/TMS): δ 0.84–2.63 (m, 32H, 3H of NHCH(CH_3)COO and 10H of DACH and 19H of COO$(CH_2)_8CH_3$), 3.28–3.51 (m, 1H, NH*CH*(CH_3)COO), 5.32–6.17 (3H, NH and NH_2); ESI-MS *m/z*: $[M + H]^+ = 552.0$ (100%).

2.3.7. Compound P5. The leaving group of compound **P5** is $CH_3(CH_2)_{10}COO^-$, so the starting material was $CH_3(CH_2)_{10}COONa$. Yield 51.8%. Anal. Calcd for $C_{21}H_{40}N_2O_4Pt$ (%): C, 43.51; H, 6.96; N, 4.83. Found (%): C, 43.56; H, 7.02; N, 4.91. IR (KBr): 3481m, 3249m, 3097m, 2924s, 2850s, 1600s(sh), 1467m, 1390m, 1339m, 1303m, 1276w, 1107w, 1039w, 837w, 7221w, 690, 459w. ^{1}H NMR (DMSO/TMS): δ 1.25–2.87 (m, 36H, 3H of NHCH(CH_3)COO and 10H of DACH and 23H of COO$(CH_2)_{10}CH_3$), 3.50–3.53 (m, 1H, NH*CH*(CH_3)COO), 5.31–6.50 (3H, NH and NH_2); ESI-MS *m/z*: $[M - H]^- = 578.2$ (100%).

2.3.8. Compound P6. The leaving group of compound **P6** is $CH_3(CH_2)_{12}COO^-$, so the starting material was $CH_3(CH_2)_{12}COONa$. Yield 49.6%. Anal. Calcd for $C_{23}H_{44}N_2O_4Pt$ (%): C, 45.46; H, 7.30; N, 4.61. Found (%): C, 45.49; H, 7.36; N, 4.51. IR (KBr): 3478m, 3243m, 3096m, 2920s, 2849m, 1559s(sh), 1446m(sh), 1421m, 722w. ^{1}H NMR (DMSO/TMS): δ 1.11–2.52 (m, 40H, 3H of NHCH(CH_3)COO and 10H of DACH and 27H of COO$(CH_2)_{12}CH_3$), 3.34–3.39 (m, 1H, NH*CH*(CH_3)COO), 5.31–6.73 (3H, NH and NH_2); ESI-MS *m/z*: $[M - H]^- = 606.2$ (100%).

2.3.9. Compound P7. The leaving group of compound **P7** is  , so the starting material was  . Yield 44.9%. Anal. Calcd for $C_{15}H_{26}N_2O_4Pt$ (%): C, 36.51; H, 5.31; N, 5.68. Found (%): C, 36.56; H, 5.38; N, 5.66. IR (KBr): 3385s(sh), 3240m, 2945s, 2865s, 1662s, 1557s, 1447m, 1407s, 1325m, 1111w, 775m, 577m, 460w. ^{1}H NMR (D_2O/TMS): δ 1.18–3.02 (m, 22H, 3H of NHCH(CH_3)COO and 10H of DACH and 9H of $(CH_2)_4CH$ (COO)), 3.75–3.97 (m, 1H, NH*CH*(CH_3)COO); ESI-MS *m/z*: $[M + H]^+ = 494.0$ (20%), $[M + H_2O + H]^+ = 512.9$ (100%).

2.3.10. Compound P8. The leaving group of compound **P8** is  , so the starting material was  . Yield 51.2%. Anal. Calcd for $C_{16}H_{28}N_2O_4Pt$ (%): C, 37.87; H, 5.56; N, 5.52. Found (%): C, 37.96; H, 5.58; N, 5.56. IR (KBr): 3420m, 3203m, 2929s, 2852m, 1654s, 1567vs, 1416vs(sh), 1278m, 1222w, 1110w, 1036w, 893w, 774m, 645m, 504m. ^{1}H

NMR (D_2O/TMS): δ 1.23–2.94 (m, 24H, 3H of NHCH(CH_3)COO and 10H of DACH and 11H of cyclohexyl), 3.87–4.05 (m, 1H, NHCH(CH_3)COO); ESI-MS *m/z*: $[M+H]^+ = 508.1$ (100%).

2.4. *Cell culture*

HepG-2, MCF-7, A549, and HCT-116 cell lines were cultured in an RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 unit mL^{-1} of penicillin, and 100 $\mu g\,mL^{-1}$ of streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.5. *Solutions*

The platinum complexes were dissolved in DMSO at a concentration of 5 mM as stock solution and diluted in culture medium at concentrations of 0, 10, 20, 50, and 100 μM as working solutions. To avoid DMSO toxicity, the concentration of DMSO was less than 0.1% (V/V) in all experiments.

2.6. *Cytotoxicity analysis*

MTT assay was carried out to evaluate the *in vitro* cytotoxicity of the resulting platinum complexes as described by Mosmann *et al.* [18]. Tumor cells were plated onto 96-well sterile plates in 100 mL of medium at a density of $4 \times 10^3 - 8 \times 10^3$ cells per well and incubated for 24 h at 37 °C in a 5% CO_2 containing incubator. The prepared compounds [PtRI], [PtSI], R1–R3, and S1–S3 were added in final concentrations ranging from 0 to 100 μM. After 48 h, 50 μL MTT in PBS (5 mg/mL) was added to each well and the plates were incubated for 3 h at 37 °C. Removing the liquid, DMSO (100 mL) was added to dissolve the MTT formazan. The OD for each well was measured on a microplate reader at a wavelength of 490 nm. All cytotoxicity tests were carried out three times in parallel; IC_{50} values were obtained from curves constructed by plotting cell survival (%) *versus* compound concentration (in μM).

3. Results and discussion

3.1. *Preparation for chiral ligands and targeted platinum complexes*

Mono-Boc protected DACH was used as starting material to prepare HP as before, since it is difficult to directly get the mono-substituted derivatives due to the equivalent reactivity of the two amino groups in DACH, and the ligand was obtained via four steps (scheme 1).

Before preparing the targeted platinum complexes, important intermediates [PtRI] were prepared by adopting a similar method as described in the literature [19]. Then $AgNO_3$ was used to remove iodide of [PtRI] to form [PtR(H_2O)]NO_3, which was *in situ* used to react with the sodium salts of the corresponding acids, respectively, to afford compounds **P1**–**P8** (scheme 2). Coupling of the propanoic acid to the amine nitrogen to form the tridentate HP ligand produces a chiral center at carbon 2 of the propanoic acid, and another chiral center is produced once the nitrogen to which C-2 is bound becomes coordinated to platinum [14].

3.2. *Characterization of the complexes*

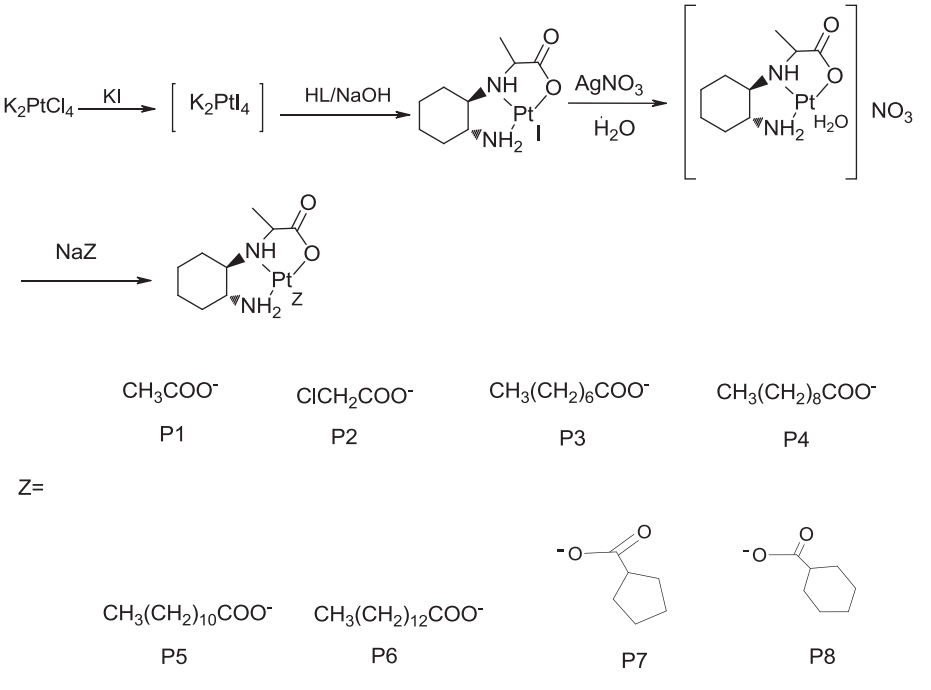
Intermediates [PtRI] and platinum complexes were characterized by IR, ^{1}H NMR, ESI-MS spectra, and microanalysis. Found values for each compound in the elemental analyses were in agreement with calculated values. The IR spectra of these complexes are similar. Pt–N coordinations were confirmed by examination of $\nu NH_2/\nu NH$ shifting to lower frequencies compared with their free amino groups. Carboxylate anions binding with Pt(II) were confirmed by examination of the C=O absorptions shifting from free carboxylic acids near 1700 cm^{-1} to bands near 1590 cm^{-1} in each case. The ^{1}H NMR spectra of the complexes are consistent with their corresponding protons both in the chemical shifts and the number of hydrogens. All prepared complexes showed a peak of $[M+H]^+$ or $[M-H]^-$ in their ESI mass spectra, which are consistent with expected molecular formula weights. Typical isotopes of Pt elements: ^{194}Pt (33%), ^{195}Pt (34%), and ^{196}Pt (25%), were found with three protonated ion isotopic peaks.

3.3. *Aqueous solubility*

Poor aqueous solubility is a severe problem for some platinum anticancer drugs in clinic use, such as cisplatin. Hence, the aqueous solubility of targeted platinum compounds was tested at 25 °C, ranging from 2.3 to 53.2 mg/mL.

3.4. *Cytotoxic studies*

In vitro cytotoxicity of targeted platinum compounds was tested by MTT colorimetric assay against HepG-2 human hepatocellular carcinoma cell, MCF-7 human breast cancer cell,



Scheme 2. Synthetic scheme for targeted platinum(II) compounds.

Table 1. Cytotoxicity of the targeted compounds against four tumor cell lines.

Compd.	IC_{50} ($\mu M\ L^{-1}$)			
	HepG-2	MCF-7	A549	HCT-116
P1	56.3	>100	>100	>100
P2	41.6	>100	>100	36.1
P3	8.6	>100	10.1	>100
P4	7.1	>100	9.6	>100
P5	38.6	>100	36.1	>100
P6	36.9	>100	38.6	>100
P7	73.1	>100	>100	>100
P8	88.6	>100	>100	>100
Carboplatin	11.0	9.2	13.1	>100
Oxaliplatin	>100	>100	>100	4.3

A549 human lung cancer cell, and HCT-116 human colorectal cancer cell, with carboplatin and oxaliplatin as positive controls.

The cytotoxicities of oxaliplatin against HepG-2, MCF-7, and A549 human lung cancer cells were tested and results showed that oxaliplatin was insensitive to them (table 1). All platinum compounds showed a certain activity against the HepG-2 cell line, but showed very low activity (<100 $\mu M\ L^{-1}$) against MCF-7 cell line. To cell lines A549 and HCT-116, a portion of the targeted complexes showed cytotoxicity and no significant regularity was found.

Among all platinum compounds, **P3** and **P4** gave lower IC_{50} values than carboplatin against HepG-2 and A549 cell lines. From **P1**–**P6**, it could be seen that all of them have taken carboxylates as the leaving group, and the length of carbon chains increases, **P1** = **P2** < **P3** < **P4** < **P5** < **P6**. Aqueous solubility of the new platinum complexes was also tested and showed a reverse order to the length of carbon chains, **P2** > **P1** > **P3** > **P4** > **P5** > **P6**. Compounds **P3** and **P4** of intermediate carbon chain length showed favorable activity against tested tumors. Speculating the reasons, if the carbon chains for the leaving group are much shorter, the solubility of platinum compounds (**P1**–**P2**, 43.5–53.2 mg/mL) may be too good to break down before forming the adducts with the tumor DNA base pairs, but when the carbon chains are much longer, the platinum compounds showed poor water solubility (**P5**–**P6**, 3.1–2.3 mg/mL), which is a common reason for platinum complexes having poor antitumor activity. **P3** and **P4**, which took $CH_3(CH_2)_6COO^-$ and $CH_3(CH_2)_8COO^-$ as leaving groups, respectively, showed medium aqueous solubility (**P3**–**P4**, 16.8–14.4 mg/mL) for medium length carbon chains, exhibiting better cytotoxicities than other platinum complexes in this study.

Compounds **P7** and **P8** showed unsatisfactory *in vitro* cytotoxicity to all tested cell lines. The reason may be that the cycloalkanes in the leaving group have steric hindrance for bonding between the platinum complexes and DNA base-pairs.

4. Conclusion

A new chiral DACH derivative, HP, was designed, synthesized, and characterized to prepare eight Pt(II) complexes. *In vitro* cytotoxicity tests showed that all compounds exhibited sensitivity to the HepG-2 cell line with **P3** and **P4**, bearing $CH_3(CH_2)_6COO^-$ and

$CH_3(CH_2)_8COO^-$ as the leaving groups, respectively, which gave better antitumor activity than carboplatin against HepG-2 and A549 cell lines. Li *et al.* [20] and Zhang *et al.* [21] reported some new platinum(II) complexes with amino acid dianion ligands and some showed selective cytotoxicities against tested tumor cell lines and are similar to the platinum compounds reported in this manuscript. Consequently, the prepared platinum compounds, especially **P3** and **P4**, may serve for further investigation.

Funding

This work is supported by the National Natural Science Foundation of China Project [grant number 21361014], [grant number 21302074] to C.Z. Gao; the National Natural Science Foundation of China Project [grant number 21362016] to B. Yang; the National Natural Science Foundation of China Project [grant number 21272041] to S.H. Gou; Doctoral Program of the Ministry of Education of PR China [grant number 20125314120007] to C.Z. Gao.

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