Synthesis, cytotoxicity and cucurbituril binding of triamine linked dinuclear platinum complexes

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The platinum complexes *trans*-[{PtCl(NH$_3$)$_2$}$_2$(μ-NH$_2$(CH$_2$)$_3$NH$_2$(CH$_2$)$_3$NH$_2$)]$^{3+}$ (CT033) and the corresponding N4-dimethyl linked analogue *trans*-[{PtCl(NH$_3$)$_2$}$_2$(μ-NH$_2$(CH$_2$)$_3$N(Me)$_2$(CH$_2$)$_3$NH$_2$)]$^{3+}$ (CT233) have been synthesised, and their cytotoxicity, ability to bind cucurbit[7,8]uril (Q[7,8]) and reaction with cysteine studied. Both platinum complexes show good activity in the L1210 cell line and maintain their activity in the corresponding cisplatin L1210/DDP cell line. However, the N4-dimethyl analogue CT233 is approximately 50-times less active than the CT033 complex. This suggests that the insertion of a positive charge into the linking ligand may not, *per se*, be responsible for the higher cytotoxicity generally observed for dinuclear platinum complexes linked by polyamines. The upfield shifts of the resonances from the methylene protons in the linking triamine ligand observed in the ^{1}H NMR spectra of either CT033 and CT233 upon addition of either Q[7] or Q[8] indicate that the cucurbituril is positioned over the linking ligand. However, the results show that the protonated secondary amine in CT033 acts as a barrier to encapsulation, with the Q[7,8] being positioned over only one propyl-arm at a time. Alternatively, the entire triamine linking ligand of CT233 is fully encapsulated within the Q[7,8] cavity. Encapsulation by Q[7,8] was found to reduce the rate of reaction of CT033 and CT233 with the thiol containing amino acid cysteine, with a greater rate reduction observed for CT233. These results are consistent with the NMR results of the Q[7,8] binding studies of the two platinum complexes. For CT033 encapsulated in Q[7,8], one of the two platinum centres is completely exposed to the solvent, whereas, for CT233 both platinum centres are simultaneously positioned within the portals of the cucurbit[*n*]uril, thereby, affording greater protection.

Introduction

Multinuclear platinum complexes, developed by Farrell and co-workers,[1–4] where two or three platinum coordination centres are linked by aliphatic amine chains, have shown excellent potential as anti-cancer agents.[1–6] While the multinuclear complexes are highly cytotoxic, and retain their activity in cisplatin resistant cell lines,[2] they have several drawbacks that could limit their clinical application. They are highly toxic, *e.g.* clinical trials have concluded that the maximum tolerated dose (MTD) for the trinuclear platinum complex BBR3464 is as low as 0.9 mg m^{-2}, compared to >60 mg m^{-2} for cisplatin.[7] Furthermore, most of the administered drug binds to thiol containing plasma proteins and is subsequently degraded into non-active metabolites.

We have proposed that encapsulation of multinuclear complexes in macrocyclic molecules called cucurbiturils could protect the platinum complexes from reaction with thiol containing blood proteins.[8] Cucurbiturils (see Fig. 1) are composed of glycoluril monomer units, and range in size from 5 to 10 units (Q[5]–Q[10]).[9–15] Cucurbiturils have the ability to form inclusion complexes with various molecules, such as diamines.[9] The hydrophobic cavity of cucurbituril allows the favourable binding of non-polar sections of a guest molecule, while the carbonyl-rimmed portals

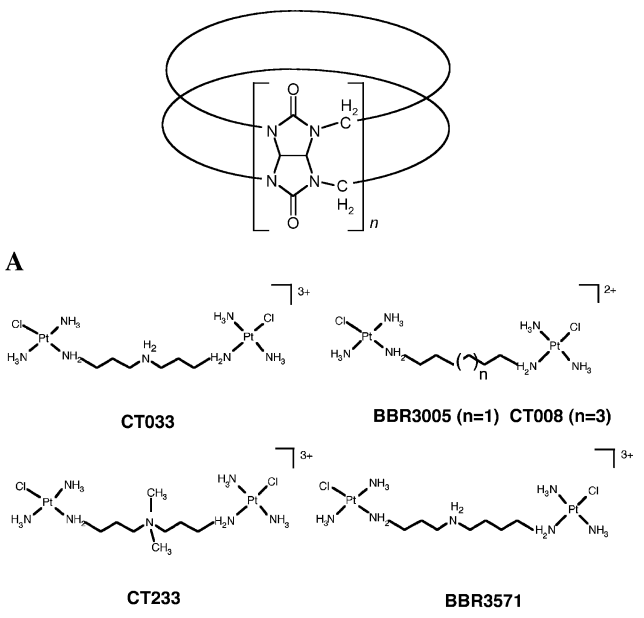

Fig. 1 (A) The chemical structure of cucurbit[*n*]uril, where $n = 7$ and 8, and (B) the structure of the dinuclear platinum complexes CT033, CT233, BBR3005, CT008 and BBR3517.

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provide hydrogen bonding potential and favourable electrostatic interactions for cationic groups.[9,16] We have demonstrated that encapsulation in Q[7] and Q[8] significantly decreases the rate of the reaction of *trans*-[{PtCl(NH$_3$)$_2$}$_2$(μ-NH$_2$(CH$_2$)$_8$NH$_2$)]$^{2+}$ (CT008) with cysteine.[8] For example, after 500 min only 20% of the platinum complex remains unreacted and intact in the absence of cucurbituril, whereas with the addition of Q[7] or Q[8], 70% and 85% of the drug remain intact respectively. From NMR studies, it was determined that the cucurbituril was positioned over the entire linking ligand, thereby protecting both platinum centres from reaction with cysteine. This was most notable for Q[8] where significant folding of the octamethylene chain was observed.

While the results of the encapsulation of dinuclear platinum complexes linked by diamines (such as 1,8-octanediamine in *trans*-[{PtCl(NH$_3$)$_2$}$_2$(μ-NH$_2$(CH$_2$)$_8$NH$_2$)]$^{2+}$(CT008)) are encouraging, it has been demonstrated that complexes linked by polyamines (*e.g.* spermidine and spermine) are considerably more cytotoxic than their diamine counterparts.[2,17] However, host–guest binding studies from the groups of Mock, Kim and Steinke have shown that a protonated secondary amine provides a barrier to encapsulation.[18–20] This is due to the high-energy cost of placing the cation inside the hydrophobic cavity of the cucurbituril, most likely because of the energy cost of desolvating the cation. Consequently, for a dinuclear platinum complex linked by a triamine, *e.g.* dipropylenetriamine, the cucurbituril would bind only over one propyl group (but "shuttle" between the two propyl-arms), and thereby, leave one platinum centre exposed to the solvent and open to attack by the thiol containing proteins in the blood plasma.

In this study we have examined the binding of Q[7] and Q[8] to *trans*-[{PtCl(NH$_3$)$_2$}$_2$(μ-NH$_2$(CH$_2$)$_3$NH$_2$(CH$_2$)$_3$NH$_2$)]$^{3+}$ and the corresponding *N*4-dimethyl linked analogue *trans*-[{PtCl(NH$_3$)$_2$}$_2$(μ-NH$_2$(CH$_2$)$_3$N(Me)$_2$(CH$_2$)$_3$NH$_2$)]$^{3+}$ (see Fig. 1). The former of these platinum complexes is the triamine derivative of the prototype dinuclear complex studied by Farrell and co-workers, *trans*-[{PtCl(NH$_3$)$_2$}$_2$(μ-NH$_2$(CH$_2$)$_6$NH$_2$)]$^{2+}$ (BBR3005),[2,21,22] while the latter platinum complex allows the examination of the effect that converting a secondary ammonium ion to a quaternary ion has on the cucurbituril binding properties. In addition, the synthesis and cytotoxicity testing of these new dinuclear platinum complexes provides further information on the structure–activity relationship for this important class of drug.

Experimental

Materials

Potassium tetrachloroplatinate(II), *N*-(3-aminopropyl)propane-1,3-diamine, di-*tert*-butyl dicarbonate were obtained from Aldrich Chemical Company and used without further purification. Q[7] and Q[8] were synthesised as previously described,[9,10] and based upon NMR analysis were ≥99% pure. Routine solvents where purchased from APS Chemicals, and were of UniVar analytical grade. *trans*-Diamminedichloroplatinum(II) was obtained from Aldrich or prepared from potassium tetrachloroplatinate. Dowex cation and anion exchange resins were obtained from Bio-Rad Laboratories.

Instrumental methods

Elemental analyses (C, H, N) were performed by the Australian National University Microanalytical Service using a Carlo Erba 1106 Automatic analyser.

NMR spectra were recorded using a Varian Unity*plus*-400 spectrometer and analysed using Varian's VNMR software. All NMR experiments where conducted at 25 °C unless otherwise stated. ^{1}H NMR spectra were referenced to TSP (0 ppm) at 25 °C by using the residual ^{1}H signal of the respective deuterated solvent. 1D spectra were recorded with between 16 and 1024 transients.

Synthesis of polyamine ligands

***N*4-BOC dipropylenetriamine.** *tert*-Butyl bis(3-aminopropyl)-carbamate was prepared according to the procedure of Laduron *et al.*[23] In a flask fitted with a Dean–Stark trap with a condenser *N*-(3-aminopropyl)propane-1,3-diamine (4.11 g, 31.37 mmol) was dissolved in 100 mL of 4-methyl-2-pentanone (methyl *iso*-butyl ketone, MIBK) under a nitrogen atmosphere (using standard Schlenk techniques). The mixture was azeotropically distilled until no further water was produced to RT. 6.84 g (31.37 mmol) of di-*tert*-butyl dicarbonate was dissolved in minimum MIBK, and was added drop-wise over 5 min. After stirring at RT for 30 min, the solution was extracted with 10 mL of water. The solvent was removed by rotary evaporation, 50 mL of *iso*-propanol added followed by 10 mL of water and the solution stirred at 60 °C for 80 min. The bulk solvent was removed by rotary evaporation. The residue was washed three times with 100 mL of distilled water. The water phase was combined and removed by rotary evaporation to yield *tert*-butyl bis(3-aminopropyl)carbamate in high purity (>99% by ^{1}H NMR) (yield: 4.300 g, 58%), ^{1}H NMR ($CDCl_3$): δ (ppm) 0.75 (t, 4H, CH_2), 1.30 (s, 9H, CH_3), 1.56 (m, 4H, CH_2), 2.58 (t, 4H, CH_2).

Synthesis of *N*4,*N*4-dimethyldipropylenetriamine

2,2′-(Iminodipropane-3,1-diyl)bis(1*H*-isoindole-1,3(2*H*)-dione). To 5.3347 g (40.65 mmol) dipropylenetriamine was added 12.042 g (81.30 mmol) of phthalic anhydride and heated under reflux in a sand bath at 200 °C for 1 h before the condenser was removed. Heating was continued till the reaction ceased to evolve bubbles of steam (approx. 2 h). The product was crystallized from ethyl acetate, then methanol. The product was filtered, washed with acetone, and air-dried. (Yield: 5.47 g, 37%). Anal. Calc. for $C_{22}H_{21}N_3O_4$: C: 67.51%; H: 5.41%; N: 10.74%. Found: C: 67.10%; H: 5.36%; N: 10.52%. ^{1}H NMR (CD_3OD): δ (ppm) 1.78 (q, 4H, CH_2), 2.50 (t, 4H, CH_2), 3.63 (t, 4H, CH_2), 7.65 (m, 8H, phathalamide).

3-Amino-*N*-(3-aminopropyl)-*N*,*N*-dimethylpropan-1-aminium chloride, dihydrochloride. 2.0 g (5.110 mmol) of 2,2′-(iminodipropane-3,1-diyl)bis(1*H*-isoindole-1,3(2*H*)-dione) and 2.8 g (26.42 mmol) of anhydrous carbonate were added to 160 mL of toluene. To the mixture was added 1.212 mL (12.764 mmol) of dimethyl sulfate and heated under reflux overnight. The mixture was allowed to cool, then 20 mL of H_2O was added and the solution boiled for 30 min. The solvent was evaporated by rotary evaporation and the residue redissolved in 100 mL of methanol. The solution was cooled to 4 °C then filtered, and 0.50 mL

(5.11 mmol) of hydrazine hydrate was added. The solution was refluxed overnight before cooling to 4 °C and filtered. 40 mL of 1 M HCl was added and the solution boiled down to ~40 mL. The remaining solvent was removed to dryness by rotary evaporation, leaving the crude product. The final product was purified on a DOWEX cation exchange column (10 mm × 20 cm). The column was prepared for use by washing with 6 M HCl (250 mL), then 0.5 M HCl (250 mL) and finally distilled water until the pH was about 6. The crude compound was dissolved in a minimum of 0.2 M HCl and was loaded onto the column. The column was washed with 500 mL 0.2 M HCl, 500 mL 0.5 M HCl, with the final product in 0.5 M HCl. The solvent was removed by rotary evaporation leaving a yellow oily residue. (Yield: 0.900 g, 67%). ^{1}H NMR (CD_3OD): δ (ppm) 2.25 (m, 4H, CH_2), 3.13 (t, 4H, CH_2), 3.19 (s, 6H, CH_3), 3,51 (t, 4H, CH_2).

3-Amino-*N*-(3-aminopropyl)-*N*,*N*-dimethylpropan-1-aminium triflate. 0.350 g (1.24 mmol) of 3-amino-*N*-(3-aminopropyl)-*N*,*N*-dimethylpropan-1-aminium chloride, dihydrochloride was dissolved in a minimum volume of water. Dowex anion exchange resin was prepared for use by washing it with 150 mL of 3 M NaOH. The resin was washed with approx. 500 mL water or until the eluant was ~pH 7.0. The compound was then loaded onto the resin, and left for 10 min, before washing with 3 × 30 mL volumes of water. The combined washings were then titrated with 1.0 g of triflic acid dissolved in 5 mL of water, until pH reached ~9.5. The compound was freeze-dried. (Yield: 0.345 g, 86%).

Synthesis of platinum complexes

{Bis[*trans*-diamminechloroplatinum(II)](μ-[*N*-(3-aminopropyl)-propane-1,3-diamine])} dichloride, hydrochloride (CT033). CT033 was prepared by a method based on that of Qu and Farrell.[24] 0.2015 g (1.186 mmol) of $AgNO_3$ was dissolved in 3 mL of DMF and added drop-wise to a stirred solution of 0.3675 g (1.22 mmol) of *trans*-diamminedichoroplatinum(II) in 10 mL DMF, and stirred for 18 h in the absence of light under a dry nitrogen atmosphere. The mixture was filtered through a 0.5 μm PETE syringe filter, and cooled to –20 °C in a 3 : 1 ice : NaCl (w/w) bath. 0.1316 g (0.569 mmol) of *tert*-butyl bis(3-aminopropyl)carbamate in 3 mL of DMF was added drop-wise over 10 min. The solution was stirred at –20 °C for 3 h, then at RT for 1 h, under anhydrous conditions. The solvent was removed *in vacuo*, and the product stirred in 30 mL of ether : acetone (1 : 1 v/v) overnight. The supernatant liquid was pippetted off and the solid dissolved in a minimal quantity of MeOH. 5 mL of conc. HCl was added and the solution stirred over 72 h. The mixture was cooled to 4 °C overnight and then filtered. The resulting yellowish solid was washed with 2 × 10 mL volumes of acetone, then ether, then dried *in vacuo* to give CT033 as a yellowish solid. (Yield: 0.342 g 78%). Anal. Calc. for $C_6H_{30}N_7Cl_5Pt_2$: C: 9.39%; H: 3.94%; N: 12.77%. Found: C: 9.59%; H: 3.83%; N: 12.31%. ^{1}H NMR (D_2O): δ(ppm) 2.12 (m, 4H, CH_2), 2.80 (m, 4H, CH_2), 3.16 (t, 4H, CH_2).

{Bis[*trans*-diamminechloroplatinum(II)](μ-[3-amino-*N*-(3-aminopropyl)-*N*,*N*-dimethylpropan-1-ol-aminium])} trichloride (CT233). CT233 was prepared by a method based on that of the groups of Rendina[25] and Farrell.[24] 0.3220 g (1.25 mmol) of AgOTf was dissolved in 3 mL of DMF and added drop-wise to a stirred solution of 0.3783 g (1.25 mmol) of *trans*-diamminedichloroplatinum(II) in 10 mL DMF, and stirred for 18 h in the absence of light under a dry nitrogen atmosphere. The mixture was filtered through a 0.5 μm PETE syringe filter, and cooled to –20 °C in a 1 : 3 NaCl : ice (w/w) bath. 2.4 mL of a 0.245 M solution (185 mg) of 3-amino-*N*-(3-aminopropyl)-*N*,*N*-dimethylpropan-1-aminium triflate was added drop-wise over 10 min. The solution was stirred at –20 °C for 3 h, then at RT for 1 h. The solution was dried *in vacuo* then washed with ether : acetone 1 : 1 then dissolved in water and freeze-dried. The resulting yellow solid was fractionally crystallized from 0.2 M HCl and MeOH, then dried to give the product as a mixed triflate–chloride salt. (Yield: 67 mg). For analysis, the product was precipitated as the PF_6^- salt by addition of excess NH_4PF_6 in a concentrated aqueous solution. Anal. Calc. for $C_8H_{34}N_7Cl_2F_{18}P_3Pt_2$: C: 8.55%; H: 3.05%; N: 8.72%, Found: C: 9.16%; H: 3.13%; N: 8.40%. The product was then dissolved in a minimum volume of H_2O. 10 g of anion exchange resin was treated with saturated NaCl solution, and then washed with excess H_2O. The solution containing the platinum compound was added to the resin and stirred for 10 min. The resin was removed by filtration and washed with 3 × 10 mL of H_2O. The combined washings were freeze-dried to give CT233 in near quantitative yield. ^{1}H NMR (D_2O): δ (ppm) 2.24 (m, 4H, CH_2), 2.80 (t, 4H, CH_2), 3.14 (s, 6H, CH_3), 3.44 (t, 4H, CH_2).

Q[*n*] encapsulation of platinum complexes

For Q[7] titrations, a stock solution of Q[7] in D_2O (~5 mM) was added in 20 μL aliquots to a solution containing 0.5 μmol of the relevant platinum complex in 0.7 mL of D_2O. Q[8] titrations where conducted by dissolving solid portions of Q[8] (~0.3 mg, 0.2 μmol) in 0.7 mL of D_2O containing ~0.5 μmol of the relevant Pt complex. 1D ^{1}H NMR spectra where recorded for each addition and NOESY and/or COSY spectra where taken at 1 : 1 platinum complex to cucurbituril ratio when assignments were not straightforward.

Cysteine reaction assays

A stock solution of buffered cysteine in simulated physiological conditions was prepared from 120 mM NaCl, 2.7 mM KCl, 26.3 mM KH_2PO_4, 123.6 mM Na_2HPO_4 and 4 mM L-cysteine in D_2O solution, as previously described.[8] The isotope pH effect was not accounted for. All solutions were gassed with argon. Free or cucurbituril encapsulated platinum drug sufficient to form 1 mM solutions was dissolved in 0.7 mL of the stock solution at 37 °C and a 1D ^{1}H NMR spectrum was taken immediately. Further spectra were taken at regular intervals.

Cytotoxicity assays

The murine leukaemia line L1210 and the cisplatin resistant subline L1210/DDP were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (CSL Ltd, Australia). The cells were maintained in a humidified incubator with 5% CO_2 in air at 37 °C and were tested routinely for mycoplasma. Cytotoxicity was determined using growth inhibition assays. The metal complex was dissolved in warm ultrapure water and then diluted to the required concentrations in complete medium. Growth inhibition

results are for a 48 h continuous exposure of the metal complex in L1210 and L1210/DDP murine leukemia cells as previously described.[26]

Results

Synthesis

The synthesis of *N*4-BOC-dipropylenetriamine is an improvement of the method utilised by Farrell *et al.* in the synthesis of *N*4-BOC-spermidine (used for BBR3571),[27] which requires selective protection of the primary amines by a protecting group which is removed and discarded once the secondary amine is protected by the BOC group. By utilising a new method of selectively protecting secondary amines in the presence of primary amines,[23] the whole reaction takes place in the one reaction vessel. The relatively cheap solvent methyl *iso*-butyl ketone replaces the more expensive ethyl-trifluoroacetate as the primary amine protecting group, and doubles as the reaction medium. The result is a more efficient reaction and a higher purity product in higher yield. The reaction scheme is shown in Fig 2. The BOC protecting group is removed after the addition of platinum centres to the primary amines, leaving the free secondary amine. This step ensures that unwanted platination of the central amine does not occur.

The platination of diamine linkers is well established, usually following the work of Farrell *et al.* in his production of the original dinuclear complexes.[24] This method uses silver nitrate and transplatin to produce a mono-labile platinum–DMF species, which is then reacted with the appropriate free base. However, it was found that the silver nitrate–DMF method needed some modification to be able to accommodate the new quaternary polyamines. In the neutralised state (required for platination), the central quaternary ammonium ion carries a charge, which requires a counter ion. As a nitrate salt, it was found that the polyamines were not soluble in DMF, which inhibited the use of the synthetic methods used by Farrell *et al.* A solution was found by incorporating aspects of the platination method used by Woodhouse and Rendina.[25] By using the triflate counterion, it was found that the quaternary polyamines were quite soluble in DMF solution. The triflate anion was added to the free base by titrating triflic acid into the hydroxide form of the free base to a pH of ~9.

Table 1 Cytotoxicity (IC_{50}) in the L1210 murine leukaemia cancer cell line and its cisplatin resistant line L1210/DDP of CT033 and CT233. IC_{50} is defined as the concentration of complex (μM) required to inhibit cell growth by 50%. The resistance factor (RF) is defined as IC_{50} resistant/IC_{50} sensitive. IC_{50} values were determined from at least two independent experiments

Complex	L1210	L1210/DDP	RF
Cisplatin	0.5	6.9	13.8
CT033	0.13	0.12	0.9
CT233	8.6	3.2	0.4
BBR3005[a]	3.0	2.4	0.8
BBR3571[a]	0.053	0.008	0.2

[a] The IC_{50} values for BBR3005 and BBR3571, using equivalent experimental conditions, were obtained from reference 2.

To alleviate any problems with mixed salts, silver triflate was used instead of silver nitrate.

Cytotoxicity

The *in vitro* cytotoxicities of CT033 and CT233 were determined against the murine leukaemia cell line L1210 and the corresponding cisplatin resistant cell line L1210/DDP. The results are summarised in Table 1. As expected, inclusion of an amine into the parent dinuclear complex *trans*-[{$PtCl(NH_3)_2$}$_2$(μ-$NH_2(CH_2)_6NH_2$)]$^{2+}$ (BBR3005) to form CT033 significantly enhanced the cytotoxicity. Interestingly, CT033 is approximately two fold less active than that reported for the corresponding dinuclear complex linked by spermidine (BBR3571).[2] CT233, where the secondary ammonium ion has been converted into a quaternary ion through the addition of methyl groups, is significantly less active than CT033, but maintains its activity in the L1210/DDP cell line.

Cucurbituril binding of CT033

Fig. 3 shows the titration of CT033 with aliquots of Q[7] and Q[8], with the changes in chemical shift of the resonances from the non-exchangeable protons summarised in Table 2. The resonances from all the methylene protons shifted upfield upon addition of either cucurbituril, with the resonance from the b protons exhibiting the greatest shift for Q[7] and the a protons for addition of Q[8].

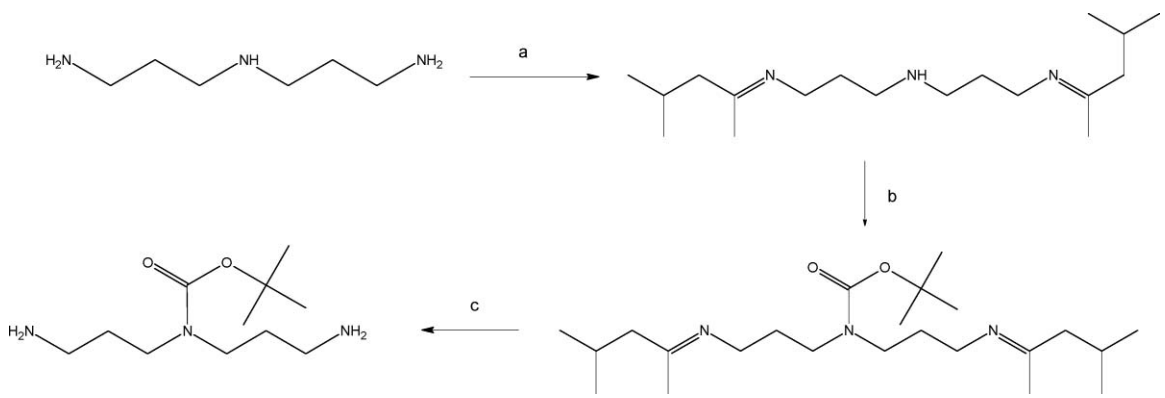

Fig. 2 Synthesis of BOC-dipropylenetriamine. Reaction conditions, one-pot (a) methyl *iso*-butyl ketone, azeotropic distillation; (b) di-*tert*-butyl dicarbonate, RT; (c) H_2O, *iso*-proponol, reflux.

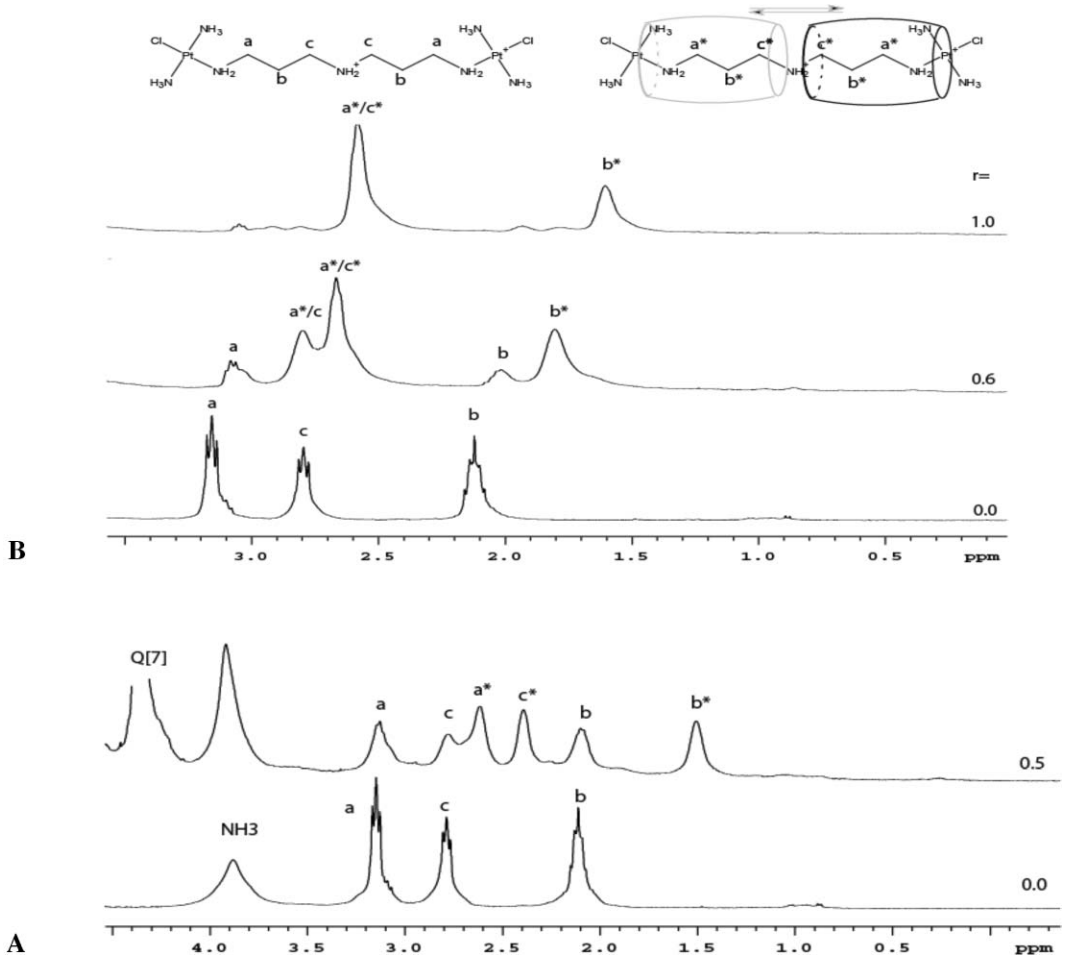

Fig. 3 ^{1}H NMR spectra of the titration of CT033 (0.7 mM in D_2O at 25 °C) with Q[7] (A) and Q[8] (B). The Q[7,8] to CT033 ratio is indicated.

Table 2 Chemical shifts of the non-exchangeable proton resonances of CT033 and Q[7]- and Q[8]-encapsulated CT033 at $r = 1$. A negative value for the change in chemical shift ($\Delta\delta$) indicates an upfield shift

	Chemical shift (ppm)		
Proton	Free CT033	Q[7]–CT033 ($\Delta\delta$)	Q[8]–CT033 ($\Delta\delta$)
a	3.14	2.61 (–0.53)	2.58 (–0.56)
b	2.11	1.51 (–0.60)	1.60 (–0.51)
c	2.79	2.39 (–0.40)	2.58 (–0.21)

At $r = 0.5$, two sets of resonances are observed for each set of methylene protons, indicating slow exchange (on the NMR time scale) between the free and Q[7,8]-encapsulated metal complex.

From the original study by Mock and Shih using diaminoalkanes,[9] and subsequent research by others using a variety of guest molecules,[16,28–30] it has been established that resonances from guest protons that are located inside the cucurbituril cavity shift upfield. Furthermore, protons that are positioned near the centre of the host cucurbituril cavity exhibit the largest upfield shifts. As the c methylene resonance shows the smallest upfield shift, and hence, is less deeply positioned in the cucurbituril cavity than the other methylene protons, it can be concluded that the central NH_2^+ group acts as a barrier to encapsulation. As only one major set of bound resonances are observed throughout the titration, it can also be concluded that the Q[7,8] "shuttles" from one propyl-arm to the other with fast exchange kinetics.

While CT033 binds Q[8] with slow exchange kinetics, a progressive change in chemical shift of the resonances of the free and bound metal complexes during the titration was noted. It suggests that, although there is slow exchange between the free and Q[8] bound platinum complex, there is another form of exchange which influences the shielding of the guest within the cucurbituril. It is concluded that this is due to a second binding mode, possibly on the outside of the Q[8], which forces the platinum complex to shift position inside the cavity. As this binding mode is in fast exchange, the average state is observed in the NMR spectrum. This proposal is supported by the observation that as the concentration of Q[8] approaches that of CT033, the shielding values approach that of the $r = 1$ complex. Further addition of Q[8] past $r = 1$ does not give further increases in shielding.

Cucurbituril binding of CT233

Fig. 4 shows the titration of CT233 with aliquots of Q[7] solution up to a 1 : 1 molar ratio. All methylene protons in the aliphatic linking ligand exhibit upfield shifts. Table 3 summarises the shift changes of all non-exchangeable protons upon full encapsulation. The largest upfield shifts are seen for the c methylene and d methyl protons, indicating that Q[7] binds centrally over the quaternary

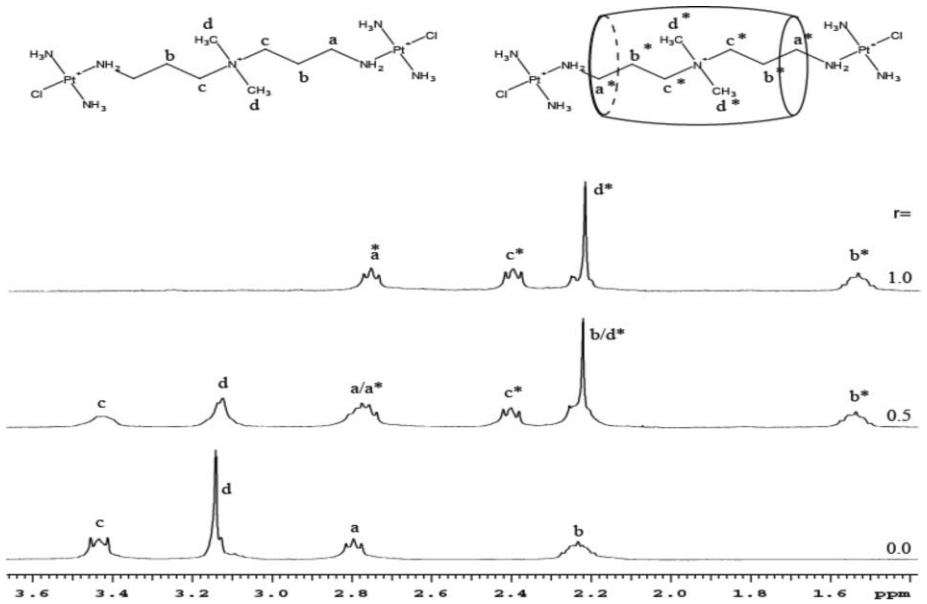

Fig. 4 ^{1}H NMR spectra of the titration of CT233 (0.7 mM in D_2O at 25 °C) with Q[7]. The Q[7] to CT233 ratio is indicated, and the structure on the right reflects the proposed binding mode.

Table 3 Chemical shifts of the non-exchangeable proton resonances of CT233 and Q[7]- and Q[8]-encapsulated CT233 at $r = 1$. A negative value for the change in chemical shift ($\Delta\delta$) indicates an upfield shift

	Chemical shift (ppm)		
Proton	Free CT233	Q[7]–CT233 ($\Delta\delta$)	Q[8]–CT233 ($\Delta\delta$)
a	2.80	2.76 (–0.04)	2.50 (–0.30)
b	2.23	1.54 (–0.69)	1.54 (–0.69)
c	3.43	2.40 (–1.03)	2.79 (–0.64)
d	3.14	2.22 (–0.92)	2.43 (–0.71)

nitrogen, with the a methylenes positioned near the Q[7] portals. A smaller upfield shift for the b methylenes and almost no shift for the a methylenes also supports this form of binding. The two sets of peaks seen at $r = 0.5$ is indicative of a slow rate of exchange between free and bound, and high binding affinity.

Fig. 5 shows the titration of CT233 at several ratios of added Q[8] solution up to a 1 : 1 molar ratio. All methylene protons in the aliphatic linking ligand exhibit upfield shifts. Table 3 summarises the shift changes of all non-exchangeable protons upon full encapsulation. The largest upfield shifts are seen for the b methylene and d methyl protons, indicating that Q[8] binds centrally over the whole molecule. Larger shifts for the a methylenes compared to the

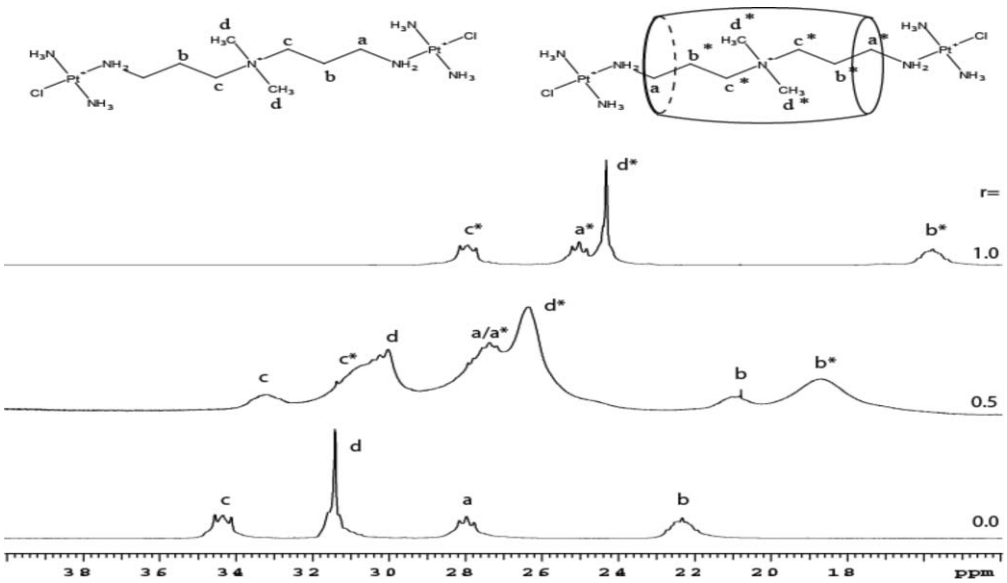

Fig. 5 ^{1}H NMR spectra of the titration of CT233 (0.7 mM in D_2O at 25 °C) with Q[8]. The Q[8] to CT233 ratio is indicated, and the structure on the right reflects the proposed binding mode.

Q[7] complex indicates a more complete encapsulation of the entire molecule. The *N*-methyl groups exhibit a smaller shift upon Q[8]-binding, compared to Q[7]-binding, whereas, the a methylenes show a larger shift upon Q[8]-binding. This indicates that the whole linking triamine ligand is folding within the Q[8] cavity. This is consistent with folding previously observed for the octamethylene linker of *trans*-[{$PtCl(NH_3)_2$}$_2$(μ-NH_2-$(CH_2)_8NH_2$)]$^{2+}$ with Q[8].[8] Alternatively, due to the significantly smaller cavity size of Q[7], little folding can take place. The two sets of peaks seen at $r = 0.5$ is indicative of a slow rate of exchange between free and bound, however the broad, gradually shifting nature of both the "free" and "bound" intermediates (at $r = 0.5$) indicates some form of intermediate exchange process.

Reaction of cysteine with CT033 and CT233

Fig. 6 and 7 show the reaction time course of 1 : 4 molar ratios of CT033 and CT233, respectively, with cysteine in phosphate buffer at 37 °C. In both cases, the percentage of the free, intact, platinum complex was reduced to 50% ($t_{1/2}$) in approximately 5 min. The rate of reaction of both CT033 and CT233 with cysteine is considerably faster than that previously observed for *trans*-[{$PtCl(NH_3)_2$}$_2$(μ-$NH_2(CH_2)_8NH_2$)]$^{2+}$ (CT008).[8] The increased rate is consistent with previous studies where insertion of a positive charge into the linking ligand of a dinuclear complex significantly enhanced the rate of reaction with DNA.[2] However, it was proposed that the observed rate acceleration for the reaction with DNA was due to the stronger reversible DNA binding association of the more highly charged cationic complex.[2]

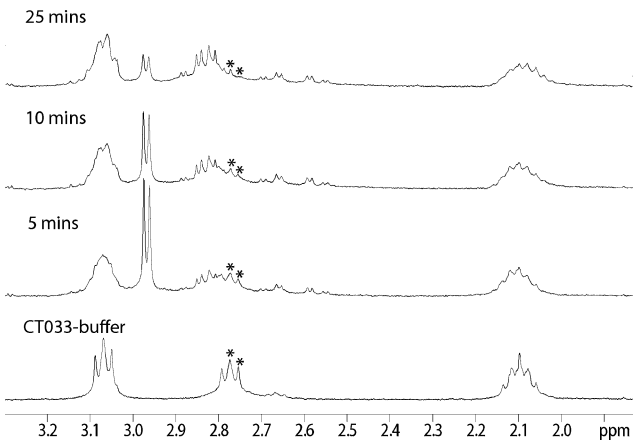

Fig. 6 ^{1}H NMR spectra of the reaction of CT033 with four equivalents of cysteine. The reaction was carried out at 37 °C in 150 mM PBS. Peaks denoted with a * in the spectrum of CT033 were used to determine the percentage of free, intact, metal complex.

Based on previous similar studies with glutathione,[31] the platinum complex is degraded into the detached amine ligand and various Pt–cysteine species in a step-wise process. Initially, the thiol group of one cysteine reacts with one platinum centre, then after another cysteine reacts with the other platinum centre the bridging amine ligand detaches. However, due to the overlap of the ^{1}H NMR resonances, no attempt was made to follow the individual species in the degradation; only the decrease of the free intact platinum complex was monitored.

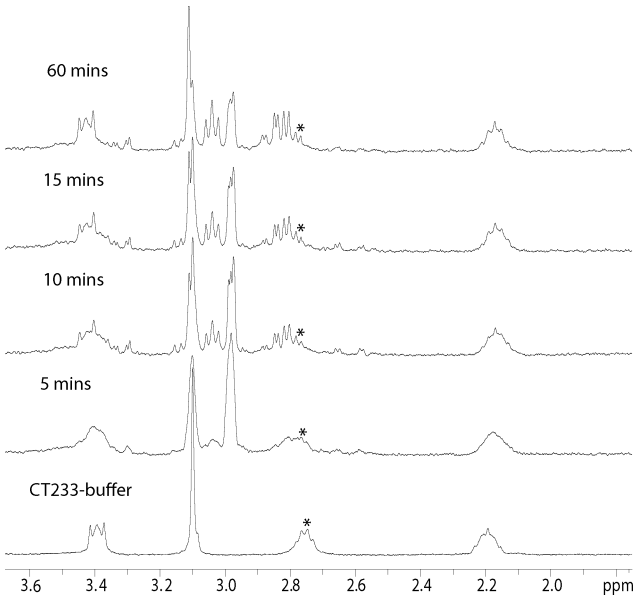

Fig. 7 ^{1}H NMR spectra of the reaction of CT233 with four equivalents of cysteine. The reaction was carried out at 37 °C in 150 mM PBS. The methylene resonances at 2.75 ppm (denoted with a *) in the spectrum of CT233 were used to determine the percentage of free, intact, metal complex.

Encapsulation in either Q[7] or Q[8] before the addition of cysteine slowed the nucleophilic reaction with the thiol group, as shown in Fig. 8 and 9. The results are summarised in Table 4.

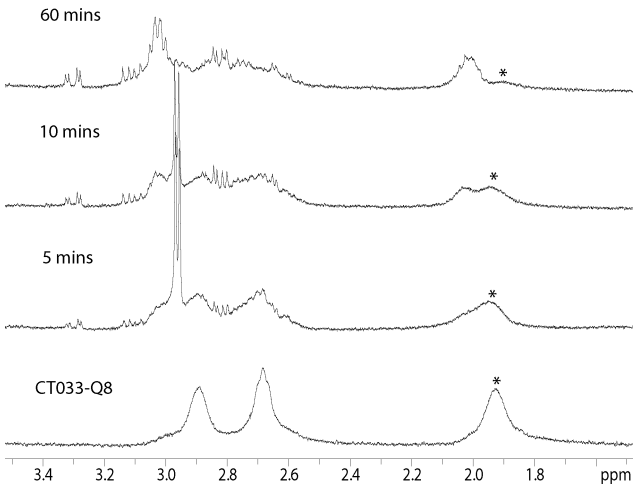

Fig. 8 ^{1}H NMR spectra of the reaction of CT033 encapsulated in Q[8] with four equivalents of cysteine. The reaction was carried out at 37 °C in 150 mM PBS. The methylene resonances at 1.93 ppm (denoted with a *) in the spectrum of CT033 were used to determine the percentage of free, intact, metal complex.

Table 4 Half-life ($t_{1/2}$), in minutes, of the reaction of CT033 and CT233 with cysteine at 37 °C. $T_{1/2}$ is defined as the time taken for the free intact platinum complex to reduce in concentration to 50%

	$t_{1/2}$ CT033/min	$t_{1/2}$ CT233/min
Free	5	5
Encapsulated in Q[7]	15	40
Encapsulated in Q[8]	10	45

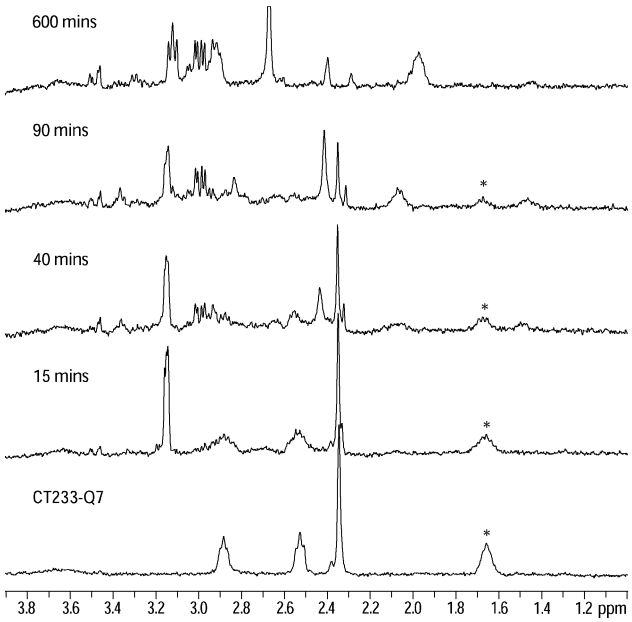

Fig. 9 ^{1}H NMR spectra of the reaction of CT233 encapsulated in Q[7] with four equivalents of cysteine. The reaction was carried out at 37 °C in 150 mM PBS. The methylene resonances at 1.65 ppm (denoted with a *) in the spectrum of CT233 were used to determine the percentage of free, intact, metal complex.

Interestingly, Q[7] and Q[8] encapsulation of CT033 only slowed the reaction with cysteine by 2–3 fold, whereas, encapsulation of CT233 slowed the reaction more significantly, with an increase in $t_{1/2}$ by 8–9 fold.

Discussion

Both CT033 and CT233 exhibit good cytotoxicity in the L1210 and L1210/DDP cell lines. As expected from the previous studies of Farrell and co-workers,[2] the inclusion of a secondary amine into the hexamethylene chain of BBR3005 to form CT033 significantly increases the cytotoxicity. However, the difference in cytotoxicity between CT033 and BBR3571 is interesting. CT033, which contains only one less methylene group in the linking chain, is two-times less active than BBR3571. CT233 is approximately 50-times less active than CT033, but it is also less active than the diamine linked BBR3005. It has been proposed that the reason for the increased cytotoxicity of multinuclear platinum complexes linked by aliphatic chains containing secondary amines is due to the combination of the cationic charge and hydrogen bonding ability of the amine.[2,27] The results of this study suggest that the increased cytotoxicity is not primarily due to the introduction of the additional positive charge.

The results of the cysteine reaction assays showed that Q[*n*] encapsulation of CT233 significantly slowed the reaction of the platinum complex with the thiol containing amino acid, whereas, encapsulation of CT033 only slowed the reaction by 2–3 fold.

The NMR results of Q[*n*] binding to CT033 showed that for both Q[7] and Q[8] the central NH_2^+ moiety acts as a barrier to encapsulation, as indicated by the relatively small shifts in the neighbouring c methylenes. Interestingly, Q[8] exerts an even smaller shielding effect on the c methylenes, presumably due to its larger cavity allowing it to move further over the platinum centre and further away from the central amine. As the largest shift observed in the Q[8]/CT033 complex is for the a methylene (closest to the platinum), it is probable that Q[8] provides better protection, than Q[7], to only one platinum centre of CT033 at a time, with the other metal centre exposed to the solution. However, this may result in the other platinum centre being more susceptible to nucleophilic attack which is consistent with the cysteine reaction assays. Symmetrical changes in chemical shift for the methylenes of both propyl-arms were seen at a 1 : 1 Q[*n*] to complex ratio, which indicates that the shuttling from one propyl-arm to the other is fast on the NMR time scale. For a non-symmetrical triamine linking ligand, the cucurbituril would be predominantly positioned over only one alkyl-arm. For example, for BBR3571 that contains a butyl- and propyl-arm on either side of the secondary amine, cucurbit[*n*]uril would predominantly bind over the butyl-arm, as the cucurbituril binding to 1,4-diaminobutane is much stronger than the corresponding propyl-analogue.[32] Furthermore, it has been established that two cucurbiturils will not "share" an amine and form a 2 : 1 complex with a triamine.

The results of this study demonstrate that the methylation of the secondary amine in the triamine linking ligand of a dinuclear platinum complex achieved the desired outcome of enabling cucurbituril to bind centrally over the entire molecule. The large shift seen for the *N*-methyl groups confirms this conclusion. The next largest shift was observed for the b methylenes, which may indicate the same form of folding as previously observed with *trans*-[{$PtCl(NH_3)_2$}$_2$(μ-$NH_2(CH_2)_8NH_2$)]$^{2+}$ and Q[8],[8] where the b methylenes are closer to the geometric centre of the cavity than the c methylenes. The binding of Q[8] to CT233 indicates the platinum centres are further within the cavity than within Q[7], as shown by the larger shielding of the a methylenes. This greater protection of the platinum centres presumably leads to the observed greater reduction in the rate of the reaction of CT233 with cysteine, compared to that with CT033.

The encapsulation of CT233 also represents a relatively rare example of a cation being positioned deep within the cucurbituril cavity. The delocalisation of the positive charge (due to the electron donating methyl groups) and the reduced thermodynamic cost of removing a quaternary amine rather than a secondary amine from water, are probable driving forces for the encapsulation.

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