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Stepwise assembly of platinum–folic acid conjugates

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Abstract

Folates and folate-like molecules represent a class of vectors for drug targeting and delivery. The folate receptor (FR) is overexpressed in many human cancers, therefore folate-cytotoxic drug conjugates can deliver therapeutic agents specifically to FR positive tumours. We have linked, directly through one of the carboxylate functionalities of the folic acid (α or γ), two different chelating arms for a cytotoxic platinum moiety, namely a diamine and a dicarboxylate. Five different derivatives were synthesised by multi-step procedures, purified and characterised; unfortunately, they resulted barely soluble in water, thereby precluding any biochemical and biological tests.
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1. Introduction

While cytotoxic agents have dominated the development of clinical cancer chemotherapy in the last half-century, growing interest is arising nowadays on alternative cytostatic drugs able to alter the cellular signals that determine cancer progression and diffusion, with the goal to core (if possible) or stabilize and treat chronically the disease. The cytotoxic approach to fight cancer suffers from severe side effects, due to the poor specificity. In particular, the hydrolysis of platinum-based drugs generates cationic complexes having electrophylic properties able to target genomic DNA (genotoxic action). The specificity of these drugs is based only on the higher proliferation index of tumour with respect to healthy cells: during mitosis, DNA is totally exposed to the electrophylic attack of the Pt-drugs in the S-phase of cycle. However, several healthy tissues have physiologically large population doubling index, such as bone mallow and gastro-intestinal tract, and their platination causes heavy side effects [1].

In recent years, the "drug targeting and delivery" approach has been developed with the aim to reduce chemotherapy-related systemic side-effects by using vectors that selectively deliver the cytotoxic agent to tumour cells, thus sparing healthy cells. Such vectors include bioactive substances, such as specific amino acids and sugars (that enter to a larger extent tumour cells by virtue of their very active metabolism) or folates and estrogen analogues (that are selectively vehiculated by the corresponding receptors often overexpressed in cancer cells). Alternatively, non-toxic, non-immunogenic and non-pyrogenic macromolecular vectors could be used for this purpose. This strategy exploits the so-called EPR (enhanced permeability and retention) effect, based on the peculiar features of tumour blood and lymphatic vessels, resulting in increased macromolecular diffusion out of the bloodstream into tumour tissue and inefficient drug drainage from the tumour.

The biological (active targeting) or macromolecular (passive targeting) vector for the cytotoxic Pt-moiety can act either as a carrier ligand or leaving group. In both cases, a spacer must be synthesized, binding the vector and ending in a coordinating arm able to link the PtX_2-unit. When the vector acts as a leaving group in the final

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complex, the coordinating functionalities are generally dicarboxylate groups. In this way, a $[Pt(NH_3)_2]^{2+}$ moiety can be delivered to DNA. The exact rate of dissociation of platinum from the pharmacophore is crucial to the overall cytotoxic properties of the conjugates. Differences in pH between healthy and tumour tissues, enzymatic activities and exchange with the carbonate/bicarbonate system concur to define the hydrolysis rate. When the vector acts as a carrier group in the final complex, it will remain coordinated to the drug upon cell penetration and DNA binding, significantly modifying the chemical-physical properties of the resulting DNA adducts (especially the steric bulk) [2].

In this context, folates and folate-like molecules represent a class of vectors for drug targeting and delivery. Folate is a generic term describing various water-soluble vitamins of the B group containing the pteroic acid nucleus in various chemical forms, including folic acid (**1**). Folates are important cofactors in C_1 transfers and are involved in two key metabolic pathways in mammalian and plant cells: DNA biosynthesis, in particular in purine and pyrimidine synthesis, and in the methylation cycle [3]. There are two well-known active transport mechanisms for folates: the reduced folate carrier (RFC) and the folate receptor (FR), respectively. The RFC has a higher affinity for L-methylfolate (reduced folate), whereas the folate receptor has a higher affinity for folic acid. The FR is overexpressed in many human cancers: for instance, in about 90% of ovarian carcinomas; moreover brain, endometrial, kidney cancers and mesothelioma are estimated to be FR-positive [4]. For these reasons folate-targeted cancer therapies can deliver therapeutic drugs specifically to FR-positive tumour cells with nanomolar affinity (Fig. 1). After the binding, the cell membrane invaginates the resulting adduct, forming an endosome. As the endosomal compartment acidifies, the drug may be released from the adduct into the cytosol [5].

Many interesting applications of the folate-drug conjugates have been reported, in particular containing toxins, low molecular weight radio- and chemo-therapeutics, immuno-therapeutic agents, liposomes with entrapped drugs, etc. [5,6].

Surprisingly, to the best of our knowledge, only two reports dealt with the application of a platinum-folate conjugate. In the first example a positive charged platinum-tetrahydrofolate complex was synthesised. This complex was a reasonably good inhibitor of L1210 dihydrofolate reductase and of the folate transport system (50% inhibition at ca. 200 μM) of L1210 cells [7]. A second reported conjugate was prepared by coupling folate to PEG_{3000} followed by its chelation with Pt(II) *via* a modified dicarboxylate ligand [8]. The folate-PEG-Pt compound showed a significantly higher uptake compared to non-targeted PEG-Pt when incubated with FR-positive M109 cells. Unfortunately, this increased Pt-content in the cells did not cause a parallel increment in Pt-DNA adducts. Moreover, cytotoxicity data further showed that the folate-PEG-Pt conjugate was 1.7-fold less effective than PEG-Pt. In fact, the folate-PEG-Pt conjugates were not freely released into the cytosol but were directed to acidic cytoplasmic endosomes and lysosomes. The release from these vesicles to the cytoplasm could be slow and inefficient. Since studies of folate-targeted pH-sensitive liposomes point to a major increase in cytosol delivery and biological activity, folate receptor guessed mediated endocytosis (FRME) may lead to an acidic vesicular compartment with reduced access to other cell compartments unless the delivery system is unstable at low pH. Therefore, the authors suggested that folate-PEG-Pt conjugates might remain trapped in acidic vesicles following FRME, thus preventing their cytotoxic activity.

In this study, we link, directly through one of the carboxylate functionalities of the folic acid (α or γ), two different chelating arms able to coordinate a cytotoxic platinum moiety, namely a diamine and a dicarboxylate. The first is an example of a carrier conjugate (cisplatin-like conjugate), while the second represents the model of a leaving conjugate (carboplatin-like conjugate) (Fig. 2).

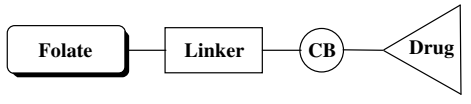


Fig. 1. Structural design of a folate-drug conjugate containing: the carrier element (**Folate**), the **Linker**, possibly with a cleavable bond (**CB**), and the therapeutic agent (**Drug**).

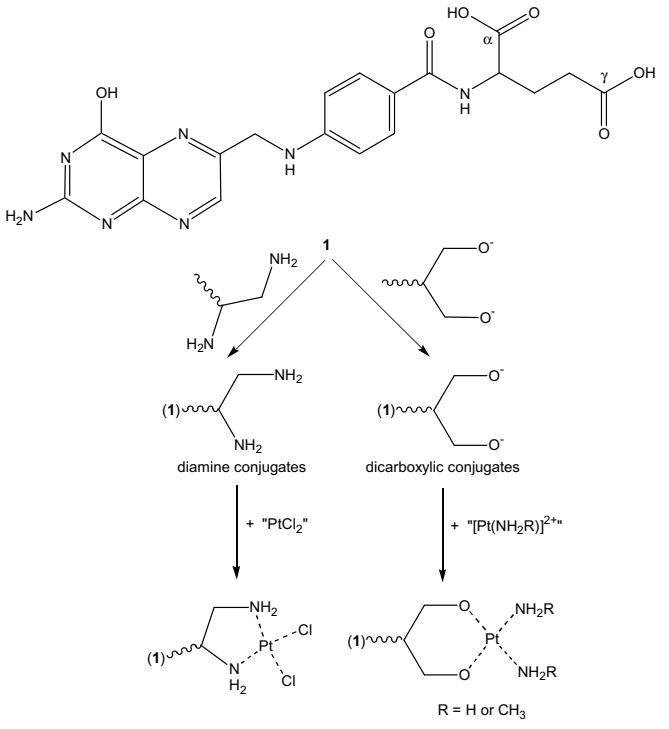


Fig. 2. Sketch of folic acid (**1**) and a general scheme of the experimental work.

2. Experimental

2.1. General procedures

$K_2[PtCl_4]$ (Johnson Matthey and Co.) and all other chemicals (Aldrich) were used without further purification. All the ligands and the low molecular weight Pt(II) complexes were characterised by multinuclear NMR spectroscopy [9]. The folic acid–Pt(II) complexes were characterised by means of thermogravimetric analysis (TGA) and inductively coupled plasma-mass spectrometry (ICP-MS). The NMR spectra for the ligands and corresponding Pt complexes were measured on a JEOL Eclipse Plus spectrometer operating at 400 MHz (^{1}H), 100.5 MHz (^{13}C) and 85.9 MHz (^{195}Pt). ^{1}H NMR and ^{13}C NMR chemical shifts were reported in parts per million referenced to residual solvent resonances. ^{195}Pt NMR spectra were recorded in DMSO-d_6, using a solution of $K_2[PtCl_4]$ in aqueous KCl as the external reference. The shift for $K_2[PtCl_4]$ was adjusted to -1628 ppm from $Na_2[PtCl_6]$ ($\delta = 0$ ppm). The thermograms were run in air atmosphere from r.t. to 1000 °C (heating speed = 10 °C/min) using a Mettler Toledo TGA/STDA851. The ICP-MS measurements were performed by means of an X5 Series ICP-MS instrument from Thermo Optek (Cinisello Balsamo, Italy). Instrument settings were optimized in order to yield maximum sensitivity for platinum. Mineralized platinum-containing material was dissolved in 2 mL of 2% HNO_3 with the addition of indium (used as the internal standard). The most abundant isotopes of platinum and indium were measured at m/z 195 and 115, respectively. Raman spectra were recorded in the 3500–50 cm^{-1} region on a Bruker RFS 100 FT-Raman Spectrophotometer with 1064 nm radiation from a Nd:YAG laser as the source of excitation. The laser power was 50 mW and the resolution was 2 cm^{-1}. IR spectra were recorded on a Bruker FTIR Equinox 55 spectrometer in the range 4000–400 cm^{-1}.

2.2. Synthesis of 2-{4-[(2-amino-4-hydroxy-pteridin-6-ylmethyl)-amino]-benzoylamino}-4-(2-tert-butoxycarbonylamino-ethylcarbamoyl)-butyric acid (**2-Boc**)

(i) *Protection of 2,3-diaminopropionic acid with di-tert-butyl dicarbonate: synthesis of 2,3-bis-tert-butoxycarbonylamino-propionic acid.* 2,3-Diaminopropionic acid monohydrochloride (1.005 g, 7.149 mmol) was dissolved in 20 ml of water and then an aqueous solution of $NaHCO_3$ (2.401 g, 28.580 mmol, 20 ml) was added. The mixture was stirred and cooled at 5 °C, then di-*tert*-butyl dicarbonate (Boc_2O, 2.003 g, 9.182 mmol) dissolved in 10 ml of dioxane was added. The mixture was kept under stirring and nitrogen steam and cooled at 0 °C. After 1 h the mixture was brought at r.t. and kept under stirring overnight. The mixture was washed twice with ethyl acetate and then the organic phase was washed twice with a saturated solution of bicarbonate. Aqueous phases were joined and acidified with 10% HCl (pH 1) and then extracted with ethyl acetate. Organic phases were joined, anhydrified with anhydrous Na_2SO_4 and evaporated to get a colourless oil. Washing with hexane resulted in a white soft powder. Yield: 850 mg, 59%. ^{1}H NMR (acetone-d_6) 6.10–6.20 (m, 2H, 2 × N*H*), 4.26 (m, 1H, C*H*, en), 3.40–3.50 (m, 2H, CH_2, en), 1.40 (s, 18H, CH_3, Boc) ppm; ^{13}C NMR (acetone-d_6) 171.89 (*C*OOH), 156.49 and 156.43 (2 × *C*(O), Boc), 78.52 and 78.38 (2 × C_{quat}, Boc), 54.54 (*C*H, en), 41.91 (*C*H_2, en), 27.75 (*C*H_3, Boc) ppm.

(ii) *Synthesis of* **2-Boc**. To stirred DMF (50 ml) at 80–90 °C folic acid **1** (0.750 g, 1.699 mmol) was slowly added and then 50 ml of DMF were added to complete the dissolution of **1**. The mixture was cooled at r.t. and CDMT (0.304 g, 1.733 mmol) was added. The mixture was cooled at 0 °C and *N*-methylmorpholine (187 μl, 1.699 mmol) was added dropwise. After 4 h mono(Boc)-ethylenediamine (0.278 g, 1.733 mmol) and *N*-methylmorpholine (187 μl, 1.699 mmol) were added. After 2 h at 0 °C the temperature was brought to r.t. and the reaction stirred for 14 h. Evaporation of the solvent resulted in a thick oil that was tritured and washed several times with cold water to remove unreacted **1** and get a yellow powder of **2-Boc**. Yield: 0.870 g, 86%. The characterisation indicated that no diadducts between **1** and mono(Boc)-ethylenediamine were formed, whereas there were only monoadduct either on α or γ carboxylic group. ^{1}H NMR (DMSO-d_6) 11.44 (br. s, 1H, H_{20}), 8.64 (s, 1H, H_7), 8.21 (d, 1H, H_{18}), 8.00–7.80 (m, 2H, H_{24} and H_{27}), 7.65 (d, 2H, H_{13} and H_{15}), 6.94 (t, 1H, H_{10}), 6.80–6.70 (m, 3H, H_2 and H_4), 6.62 (d, 2H, H_{12} and H_{16}), 4.49 (d, 2H, H_9), 4.31 (m, 1H, H_{19}), 3.09–2.96 (m, 4H, H_{25} and H_{26},), 2.32–1.85 (m, 4H, H_{21} and H_{22}), 1.34 (s, 9H, CH_3, Boc), ppm; ^{13}C NMR (DMSO-d_6) 174.40 and 174.66 (C_{20}, double signal due to α and γ functionalizations), 172.26 and 172.39 (C_{23}, double signal due to α and γ functionalizations), 166.88 (C_{17}), 161.50 (C_2), 157.25 (C_4), 156.17 (*C*(O), Boc), 154.33 (C_{8a}), 151.33 (C_{11}), 149.50 and 149.21 (C_6 and C_7), 129.56 (C_{13} and C_{15}), 128.51 (C_{4a}), 121.89 (C_{14}), 111.75 (C_{12} and C_{16}), 78.18 and 78.23 (C_{quat}, Boc), 52.54 and 53.32 (C_{19}, double signal due to α and γ functionalizations), 46.48 (C_9), 39.88 and 40.30 (C_{25} and C_{26}), 31.07 and 32.51 (C_{22}, double signal due to α and γ functionalizations), 28.76 (*C*H_3, Boc), 27.14 and 27.49 (C_{21}, double signal due to α and γ functionalizations) ppm.

2.3. Synthesis of 2-{4-[(2-amino-4-hydroxy-pteridin-6-ylmethyl)-amino]-benzoylamino}-4-[2-(2,3-bis-tert-butoxycarbonylamino-propionylamino)-ethylcarbamoyl]-butyric acid (**3**)

(i) *Deprotection of 2-{4-[(2-amino-4-hydroxy-pteridin-6-ylmethyl)-amino]-benzoylamino}-4-(2-tert-butoxycarbonylamino-ethylcarbamoyl)-butyric acid (***2-Boc***).* 2-{4-[(2-Amino-4-hydroxy-pteridin-6-ylmethyl)-amino]-benzoylamino}-4-(2-*tert*-butoxycarbonylamino-ethylcarbamoyl)-butyric acid (0.850 g, 1.456 mmol) was dissolved at r.t. in 6.7 ml of trifluoroacetic acid (TFA). After 1 h stirring at r.t. in the

dark, the solution was evaporated to dryness resulting in a thick brown oil that was tritured with diethyl ether to get a yellow powder of **2** (as CF_3COOH salt). Yield: 0.792 g, 91%. ^{1}H NMR (DMSO-d_6) 1.89–2.32 (m, 4H, H_{21} and H_{22}), 2.83–3.32 (m, 4H, H_{25} and H_{26}), 4.35 (m, 1H, H_{19}), 4.55 (d, 2H, H_9), 6.66 (d, 2H, H_{12} and H_{16}), 7.67 (d, 2H, H_{13} and H_{15}), 7.69–8.25 (m, 5H, H_2, H_4, H_{10} and H_{18}), 8.71 (s, 1H, H_7), 11.17 (br. s, 1H, H_{20}) ppm; ^{13}C NMR (DMSO-d_6) 174.33 and 174.64 (C_{23}, double signal due to α and γ functionalizations), 173.09 and 173.02 (C_{20}, double signal due to α and γ functionalizations), 167.10 (C_{17}), 159.23 and 159.58 (C_2 and C_4), 153.67 (C_{8a}), 151.30 (C_{11}), 148.70 and 148.66 (C_6 and C_7), 129.65 (C_{13} and C_{15}), 128.51 (C_{4a}), 121.89 (C_{14}), 111.80 (C_{12} and C_{16}), 52.26 and 53.48 (C_{19}, double signal due to α and γ functionalizations), 46.36 (C_9), 31.01 and 32.34 (C_{22}, double signal due to α and γ functionalizations), 36.96 and 39.21 (C_{25} and C_{26}), 26.97 and 27.06 (C_{21}, double signal due to α and γ functionalizations) ppm.

(ii) *Synthesis of* **3**. 2,3-bis-*tert*-butoxycarbonylamino-propionic acid (0.554 g, 1.82 mmol) was dissolved in DMF (50 ml) at r.t.; CDMT (0.325 g, 1.85 mmol) was added and the mixture was cooled at 0 °C; after 10 min *N*-methylmorpholine (200 μl, 1.82 mmol) was added dropwise. After 4 h **2** (1.106 g, 1.85 mmol) and *N*-methylmorpholine (0.184 g, 1.82 mmol) were added. After 2 h at 0 °C the temperature was brought to r.t. and the reaction stirred for 14 h. Evaporation of the solvent resulted in a thick oil that was tritured with cold water resulting in a yellow powder. Yield: 0.760 g, 53%. ^{1}H NMR (DMSO-d_6) 11.40 (br. s, 1H, H_{20}), 8.64 (s, 1H, H_7), 7.66 (d, 2H, H_{13} and H_{15}), 8.20–6.70 (m, 9H, H_2, H_4, H_{10}, H_{18}, H_{24}, H_{27}, H_{31}, and H_{37}), 6.62 (d, 2H, H_{12} and H_{16}), 4.48 (d, 2H, H_9), 4.33 (m, 1H, H_{19}), 3.94–2.83 (m, 7H, H_{25}, H_{26}, H_{29} and H_{30}), 2.31–1.88 (m, 4H, H_{21} and H_{22}), 1.36 (s, 18H, CH_3, Boc), ppm; ^{13}C NMR (DMSO-d_6) 174.38 and 174.65 (C_{20}, double signals due to α and γ functionalizations), 172.28 and 172.41 (C_{23}, double signals due to α and γ functionalizations), 170.76 (C_{28}), 166.90 (C_{17}), 161.41 (2 × C(O), Boc), 156.24 and 156.34 (C_2 and C_4), 152.37 (C_{8a}), 151.33 (C_{11}), 149.16 and 148.89 (C_6 and C_7), 129.68 (C_{13} and C_{15}), 128.50 (C_{4a}), 121.90 (C_{14}), 111.72 (C_{12} and C_{16}), 78.54 and 78.82 (2 × C_{quat}, Boc), 55.42 (C_{29}), 52.67 and 53.32 (C_{19}, double signal due to α and γ functionalizations), 46.47 (C_9), 42.41 (C_{30}), 38.74 and 38.89 (C_{25} and C_{26}), 31.06 and 32.58 (C_{22}, double signal due to α and γ functionalizations), 28.72 (CH_3, Boc), 27.40 and 27.47 (C_{21}, double signal due to α and γ functionalizations) ppm.

*2.4. Synthesis of cis-[(2-(4-((2-amino-4-hydroxy-pteridin-6-ylmethyl)-amino)-benzoylamino)-4-(2-(2,3-bis-tert-butoxycarbonylamino-propionylamino)-ethylcarbamoyl)-butyric acid) dichloride platinum(II)] ((**L1**)$PtCl_2$)*

(i) *Deprotection of* **3** *to give* **L1**. 2-{4-[(2-Amino-4-hydroxy-pteridin-6-ylmethyl)-amino]-benzoylamino}-4-[2-(2,3-bis-*tert*-butoxycarbonylamino-propionylamino)-ethylcarbamoyl]-butyric acid, **3** (0.748 g, 0.972 mmol) was treated at r.t. with 4.4 ml of TFA. After 3 h stirring at r.t., the solution was evaporated to dryness resulting in a thick brown oil that was tritured with diethyl ether to get a yellow powder of 2-{4-[(2-amino-4-hydroxy-pteridin-6-ylmethyl)-amino]-benzoylamino}-4-[2-(2,3-bis-*tert*-butoxycarbonylamino-propionylamino)-ethylcarbamoyl]-butyric acid di-trifluoroacetate (**L1**). Yield: 0.770 g, 99%. ^{1}H NMR (DMSO-d_6) 11.41 (br. s, 1H, H_{20}), 8.69 (s, 1H, H_7), 8.25–7.10 (m, 11H, H_2, H_4, H_{10}, H_{18}, H_{24}, H_{27}, H_{31} and H_{37}), 7.68 (d, 2H, H_{13} and H_{15}), 6.65 (d, 2H, H_{12} and H_{16}), 4.53 (s, 2H, H_9), 4.33 (m, 1H, H_{19}), 4.03–2.84 (m, 7H, H_{25}, H_{26}, H_{29} and H_{30}), 2.32–1.91 (m, 4H, H_{21} and H_{22}) ppm; ^{13}C NMR (DMSO-d_6) 174.32 and 174.64 (C_{20}, double signals due to α and γ functionalizations), 173.12 and 173.02, (C_{23}, double signals due to α and γ functionalizations), 172.81 (C_{28}), 165.92 (C_{17}), 159.17 and 159.56 (C_2 and C_4), 151.36 (C_{8a}), 151.36 (C_{11}), 149.00 and 148.78 (C_6 and C_7), 129.58 (C_{13} and C_{15}), 128.50 (C_{4a}), 121.85 (C_{14}), 111.80 (C_{12} and C_{16}), 53.48 (C_{29}), 52.30 and 50.98 (C_{19}, double signal due to α and γ functionalizations), 46.40 (C_9), 36.96, 37.03 and 38.38 (C_{25}, C_{26} and C_{30}), 31.01 and 32.33 (C_{22}, double signal due to α and γ functionalizations), 27.06 and 27.08 (C_{21}, double signal due to α and γ functionalizations) ppm. ^{19}F NMR (DMSO-d_6) −74.26 ppm.

(ii) *Synthesis of* (**L1**)$PtCl_2$. **L1** (0.830 g, 1.041 mmol) was dissolved in water (40 ml) at 65 °C and then a solution of $K_2[PtCl_4]$ (0.432 g, 0.306 mmol) in water (5 ml) was added and the pH value was adjusted to 5–6 with 0.1N aqueous KOH (the pH of the mixture tended to 1–2 because of the progressing reaction but it had to be kept from the region of hydroxocomplexes, *i.e.* at basic pH). The resulting light brown precipitate was washed with cold water and dried under vacuum (0.227 g, yield 85%). ^{1}H NMR (DMSO-d_6) 11.55 (br. s, 1H, H_{20}), 8.68 (s, 1H, H_7), 7.68 (d, 2H, H_{13} and H_{15}), 8.25–6.97 (m, 11H, H_2, H_4, H_{10}, H_{18}, H_{24}, H_{27}, H_{31}, H_{37}), 6.56 (d, 2H, H_{12} and H_{16}), 4.49 (m, 2H, H_9), 4.33 (m, 1H, H_{19}), 4.02–2.80 (m, 7H, H_{25}, H_{26}, H_{29} and H_{30}), 2.32–1.91 (m, 4H, H_{21} and H_{22}), ppm; ^{13}C NMR (DMSO-d_6) 174.48 and 174.65 (C_{20}, double signals due to α and γ functionalizations), 174.32 (C_{28}), 172.50 and 173.50 (C_{23}, double signals due to α and γ functionalizations), 167.00 (C_{17}), 157.12 and 161.35 (C_2 and C_4), 154.38 (C_{8a}), 151.36 (C_{11}), 149.00 and 149.28 (C_6 and C_7), 129.65 (C_{13} and C_{15}), 128.52 (C_{4a}), 121.72 (C_{14}), 111.75 (C_{12} and C_{16}), 53.48 (C_{29}), 52.31 and 52.59 (C_{19}, double signal due to α and γ functionalizations), 46.47 (C_9), 36.95, 38.53 and 50.16 (C_{25}, C_{26} and C_{30}), 31.02 and 31.14 (C_{22}, double signal due to α and γ functionalizations), 27.10 and 27.25 (C_{21}, double signal due to α and γ functionalizations) ppm. ^{195}Pt NMR (DMSO-d_6) −3233 and −3243 ppm (these signals correspond to the complex (**L1**)PtCl(DMSO)). TGA: Pt and K_2O residue 27.71%, calc. Pt and K_2O residue 27.72%; ICP-MS: Pt content 22.25%, calc. Pt content 22.33%.

2.5. Synthesis of the protected carboxylic ligands

2.5.1. Synthesis of 2-(4-{4-[(2-amino-4-oxo-3,4-dihydropteridin-6-ylmethyl)-amino]-benzoylamino}-4-carboxybutyrylamino)-malonic acid diethyl ester (*4*)

Folic acid **1** (0.906 g, 2.052 mmol) was dissolved in 80 ml DMF in a 100 ml flask, under stirring at 90 °C. After dissolution of folic acid, the mixture was cooled at r.t. and CMDT (0.353 g, 2.012 mmol) was added. The mixture was cooled to 0 °C and *N*-methylmorpholine (226 μl, 2.052 mmol) was added. The mixture reacted under stirring at 0 °C for 4 h and then 2-amino-malonic acid diethyl ester (0.426 g, 2.012 mmol) and *N*-methylmorpholine (226 μl, 2.052 mmol) dissolved in 10 ml DMF were added. The mixture reacted under stirring at 0 °C for 2 h and then for 14 h at r.t. to get a yellow mixture. DMF was removed under reduced pressure and the residue was washed with water (to remove unreacted folic acid), ethanol and diethyl ether. Yield: 0.503 g, 41%. ^{1}H NMR (DMSO-d_6) δ (ppm): 11.44 (s, 1H, H_{20}), 8.81–8.69 (m, 2H, NH_{24} and NH_{18}), 8.65 (s, 2H, H_7), 8.17–8.08 (m, 2H, NH_{10} and H_3), 7.65 (d, 2H, H_{13} and H_{15}), 6.96 (m, 3H, H_2 and H_4), 6.64 (d, 2H, H_{12} and H_{16}), 5.06 (d, 1H, H_{25}), 4.49 (d, 2H, H_9), 4.35 (m, 1H, H_{19}), 4.19 (m, 4H, H_{27} and H_{30}), 2.32 (m, 2H, H_{22}), 1.89 (m, 2H, H_{21}), 1.18 (t, 6H, H_{28} and H_{31}); ^{13}C NMR (DMSO-d_6) δ (ppm): 174.65 and 174.34 (C_{20}, double signal due to α and γ functionalizations), 172.73 and 172.64 (C_{23}, double signal due to α and γ functionalizations), 167.00 (C_{26} and C_{29}), 166.92 (C_{17}), 161.40 (C_4), 157.16 (C_2), 154.28 (C_{8a}), 151.40 (C_{11}), 149.26 and 149.07 (C_6 and C_7), 129.63 (C_{13} and C_{15}), 128.52 (C_{4a}), 121.86 (C_{14}), 111.74 (C_{12} and C_{16}), 62.30 (C_{27} and C_{30}), 56.74 (C_{25}), 52.79 and 52.72 (C_{19}, double signal due to α and γ functionalizations), 46.48 (C_9), 32.01 and 30.99 (C_{22}, double signal due to α and γ functionalizations), 27.42 and 27.05 (C_{21}, double signal due to α and γ functionalizations), 14.41 (C_{28} and C_{31}).

2.5.2. Synthesis of 2-[3-(4-{4-[(2-Amino-4-oxo-3,4-dihydro-pteridin-6-ylmethyl)-amino]-benzoylamino}-4-carboxy-butyrylamino)-propyl]-malonic acid di-tert-butyl ester (*5*)

Folic acid **1** (0.602 g, 1.364 mmol) was dissolved in 70 ml DMF in a 100 ml flask, under stirring at 90 °C. After dissolution of **1**, the mixture was cooled at r.t. and CMDT (0.245 g, 1.394 mmol) was then added. The mixture was cooled to 0 °C and *N*-methylmorpholine (150 μl, 1.364 mmol) was added. The mixture reacted under stirring at 0 °C for 4 h and then 2-(3-amino-propyl)-malonic acid di-*tert*-butyl ester (0.381 g, 1.394 mmol) and *N*-methylmorpholine (150 μl, 1.364 mmol) dissolved in 5 ml DMF were added. The mixture reacted under stirring at 0 °C for 2 h and then for 14 h at r.t.. After that time the mixture was pale yellow. DMF was removed under reduced pressure and the residue was washed with water (to remove unreacted folic acid), ethanol and diethyl ether. Yield: 0.808 g, 85%. ^{1}H NMR (DMSO-d_6) δ (ppm): 11.45 (br. s, 1H, H_{20}), 8.65 (H_7), 8.22–7.87 (m, 3H, H_{24}, H_{18} and H_{10}), 7.65 (d, 2H, H_{13} and H_{15}), 6.95 (m, 3H, NH_2 and H_4), 6.64 (d, 2H, H_{12} and H_{16}), 4.49 (d, 2H, H_9), 4.37 (m, 1H, H_{19}), 3.18 (t, 1H, H_{28}), 3.01 (m, 2H, H_{25}), 2.33–1.92 (m, 4H, H_{21} and H_{22}), 1.63 (m, 2H, H_{27}), 1.39–1.38 (m, 20H, H_{26}, H_{31}, H_{32}, H_{33}, H_{36}, H_{37}, and H_{38}); ^{13}C NMR (DMSO-d_6) δ (ppm): 174.62 and 174.40 (C_{20}, double signal due to α and γ functionalizations), 172.12 and 171.96 (C_{23}, double signal due to α and γ functionalizations), 168.81 (C_{29} and C_{34}), 166.80 (C_{17}), 161.39 (C_4), 157.14 (C_2), 154.32 (C_{8a}), 151.32 (C_{11}), 149.25 and 149.12 (C_6 and C_7), 129.63 (C_{13} and C_{15}), 128.51 (C_{4a}), 122.00 (C_{14}), 111.74 (C_{12}, C_{16}), 81.28 (C_{30}, C_{35}), 53.32 (C_{28}), 52.80 and 51.31 (C_{19}, double signal due to α and γ functionalizations), 46.49 (C_9), 38.55 (C_{25}), 32.64 and 32.62 (C_{22}, double signal due to α and γ functionalizations), 31.12 (C_{26}), 28.08 (C_{31}, C_{32}, C_{33}, C_{36}, C_{37} and C_{38}), 27.18 and 27.17 (C_{21}, double signal due to α and γ functionalizations), 26.09 (C_{27}).

2.6. Synthesis of Pt(II) complexes of the carboxylic ligands

2.6.1. Synthesis of cis-[$(NH_2R)_2$ PtI_2] (R = H, Me)

K_2PtCl_4 (0.415 g, 1.000 mmol) was dissolved in water (4 ml) and heated over a sand bath at 40 °C under stirring. An aqueous solution of KI (0.996 g, 6.000 mmol of KI in 2 ml of water) was added to the mixture in the dark. After 30 min, the mixture was filtered and an aqueous solution (3.330 mmol, 2 M) of NH_3 or NH_2CH_3 was added to the filtrate; this induced the immediate precipitation of fine yellow crystals of *cis*-[$(NH_3)_2PtI_2$]. The precipitate was filtered and washed with water, ethanol and diethyl ether. Yields: 90–96%.

2.6.2. Synthesis of cis-[diammino(potassium 2-{4-[(2-amino-4-oxo-3,4-dihydro-pteridin-6-ylmethyl)-amino]-benzoylamino}-4-(dicarboxymethyl-carbamoyl)-butyrato)platinum(II)], (*L2*)Pt(NH_3)$_2$

(i) *Deprotection of* **4**. 2-(4-{4-[(2-Amino-4-oxo-3,4-dihydro-pteridin-6-ylmethyl)-amino]-benzoylamino}-4-carboxy-butyrylamino)-malonic acid diethyl ester **4** (0.379 g, 0.634 mmol) was dissolved in 20 ml water by adding a stoichiometric amount of KOH (0.071 g, 1.268 mmol) and getting **L2** as potassium salt.

(ii) *Synthesis of* (**L2**)Pt(NH_3)$_2$. *cis*-[$(NH_3)_2PtI_2$] (0.300 g, 0.621 mmol) was added to a solution of $AgNO_3$ (0.207 g, 1.218 mmol) in water (~30 ml) and the mixture was stirred at 40 °C overnight in the dark. It was then filtered to remove AgI and **L2** (as potassium salt) was added to the filtrate. Immediately the precipitation of an orange compound started. The precipitate was isolated by centrifugation and washed with water, ethanol and diethyl ether and dried under vacuum. Yield: 0.421 g, 84%. TGA: Pt and K_2O residue 29.86%, calc. Pt and K_2O residue 29.97%; ICP-MS: Pt content 24.06%, calc. Pt content 24.15%.

*2.6.3. Synthesis of cis-[di(methylamine)(potassium 2-{4-[(2-amino-4-oxo-3,4-dihydro-pteridin-6-ylmethyl)-amino]-benzoylamino}-4-(dicarboxymethyl-carbamoyl)-butyrato)platinum(II)], (**L2**)Pt(NH_2CH_3)$_2$*

(i) *Deprotection of* **4**. The reaction was carried out as described for (**L2**)Pt(NH_3)$_2$.

(ii) *Synthesis of* (**L2**)*Pt*(NH_2CH_3)$_2$. *cis*-[(NH_2CH_3)$_2$-PtI_2] (0.161 g, 0.317 mmol) was added to a solution of $AgNO_3$ (0.105 g, 0.620 mmol) in water (~30 ml) and the mixture was stirred at 40 °C overnight in the dark. It was then filtered to remove AgI and a stoichiometric quantity of **L2** (as potassium salt) was added to the filtrate. Slowly a suspension of an orange compound started. Water is removed under reduced pressure and the residue was washed with water, ethanol and diethyl ether and dried under vacuum. Yield: 0.425 g, 82%. TGA: Pt and K_2O residue 29.07%, calc. Pt and K_2O residue 28.98%; ICP-MS: Pt content 23.42%, calc. Pt content 23.34%.

*2.6.4. Synthesis of cis-[diammino(potassium 2-{4-[(2-amino-4-oxo-3,4-dihydro-pteridin-6-ylmethyl)-amino]-benzoylamino}-4-(4,4-dicarboxy-butylcarbamoyl)-butyrate)platinum(II)], (**L3**)Pt(NH_3)$_2$*

(i) *Deprotection of* **5**. 2-[3-(4-{4-[(2-amino-4-oxo-3,4-dihydro-pteridin-6-ylmethyl)-amino]-benzoylamino}-4-carboxy-butyrylamino)-propyl]-malonic acid di-*tert*-butyl ester **5** (0.578 g, 0.830 mmol) reacted with trifluoroacetic acid (TFA, 20 ml) for 1 h. Excess TFA was removed under reduced pressure and the residue was washed with diethyl ether. The resulting acid was dissolved in water and KOH (0.093 g, 1.66 mmol) was added to get **L3** (as potassium salt).

(ii) *Synthesis of* (**L3**)*Pt*(NH_3)$_2$. *cis*-[(NH_3)$_2PtI_2$] (0.393 g, 0.814 mmol) was added to a solution of $AgNO_3$ (0.263 g, 1.546 mmol) in water (~20 ml) and the mixture was stirred at 40 °C overnight in the dark. It was then filtered to remove AgI and **L3** (as potassium salt) was added to the filtrate. Immediately the precipitation of an orange compound started. The precipitate was isolated by centrifugation and washed with water, ethanol and diethyl ether and dried under vacuum. Yield: 0.602 g, 87%. TGA: Pt and K_2O residue 28.61%, calc. Pt and K_2O residue 28.50%; ICP-MS: Pt content 23.03%, calc. Pt content 22.96%.

*2.6.5. Synthesis of cis-[di(methylamine)(potassium 2-{4-[(2-amino-4-oxo-3,4-dihydro-pteridin-6-ylmethyl)-amino]-benzoylamino}-4-(4,4-dicarboxy-butylcarbamoyl)-butyrate)platinum(II)], (**L3**)Pt(NH_2CH_3)$_2$*

(i) *Deprotection of* **5**. The reaction was carried out as described for (**L3**)Pt(NH_3)$_2$.

(ii) *Synthesis of* (**L3**)*Pt*(NH_2CH_3)$_2$. *cis*-[(NH_2CH_3)$_2$-PtI_2] (0.205 g, 0.403 mmol) was added to a solution of $AgNO_3$ (0.134 g, 0.789 mmol) in water (~30 ml) and the mixture was stirred at 40 °C overnight in the dark. It was then filtered to remove AgI and a stoichiometric quantity of **L3** (as potassium salt) was added to the filtrate. Immediately the precipitation of an orange compound started. The precipitate was isolated by centrifugation and washed with water, ethanol and diethyl ether and dried under vacuum. Yield: 0.304 g, 86%. TGA: Pt and K_2O residue 27.49%, calc. Pt and K_2O residue 27.59%; ICP-MS: Pt content 22.17%, calc. Pt content 22.22%.

3. Results and discussion

3.1. General remarks

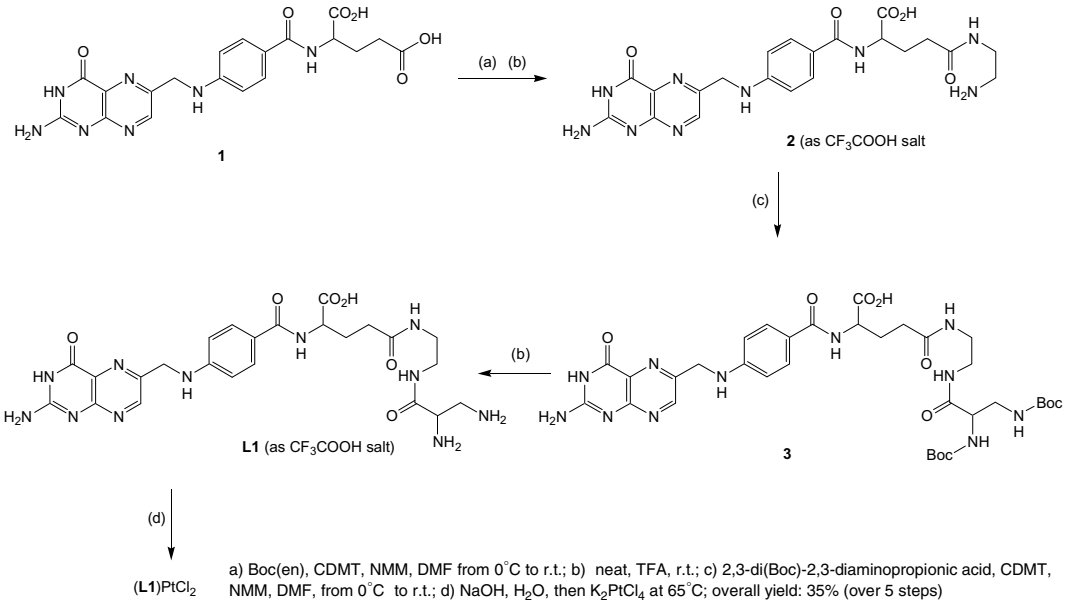
Folic acid **1** (also known as pteroylglutamic acid) can be considered a conjugated pterin therein three subunits can be recognized: 6-methylpterin, 4-aminobenzoic acid and glutamic acid. In particular, the distal glutamyl residue embodies two carboxylates (*i.e.*, α- and γ-) which will serve as handles for further synthetic manipulations (Fig. 2). It has been suggested that the receptor binding properties of folic acid are retained when derivatized *via* its γ-carboxylate, and somewhat weakened when α-carboxylate is involved [10]. However, this matter is nowadays controversially discussed [11]. Accordingly, recent studies of ^{99m}Tc-derivatives of folic acid indicate that the receptor recognition and the following internalisation of these derivatives are almost the same for both α- and γ-derivatized compounds as well as for pteroic acid conjugates [11b]. Taken together, the foregoing results indicate that the regioselectivity (α- versus γ-functionalization) could represent an unresolved but not dramatic problem in the preparation of folate-drug conjugates. In the event, the product profile for both steps (activation and coupling) is exceedingly complex [12]. Besides α- and γ-mono activated derivatives, a canonical peptide coupling protocol using *N*,*N*′-dicyclohexylcarbodiimide (DCC) in the presence of an activator (*N*-hydroxysuccinimide, HOSu) generates various by-product like bis-activated derivatives and anhydrides. As a consequence, several undesirable side-products are formed in the subsequent reaction with nucleophiles and coupling occurring at the α-carboxylate proceeds with complete racemization. Furthermore, it has been reported that isomerization between α-conjugates and γ-conjugates frequently occurs through the agency of DCC [13]. Accordingly, separation of these mixtures [10a,14] is troublesome and, indeed, some of the biological results reported in the literature have been obtained on mixtures of all the possible compounds. Nevertheless, under optimized conditions, reaction between folic acid and primary amines gave a mixture of α- or γ-conjugates and unreacted folic acid with no apparent bi-conjugates [10a,15]. In any case, the γ-derivative is claimed to be the main product of the reaction [10a,14,16].

We used coordinating arms of different length, either carrying (diamine) group or leaving (malonate) group. In particular, we synthesised one diamine conjugate (**L1**) to realize a cisplatin-analogue (**L1**)$PtCl_2$ able to exploit the folate receptor-mediated endocytosis (FRME) to deliver platinum once inside the cells. Then, we synthesised two dicarboxylic conjugates (**L2** and **L3**), which differ in the length of the

coordinating arm, to realize metal chelates not only able to take advantage of FRME but also to directly release platinum moiety after hydrolysis of the folic leaving ligand.

3.2. Synthesis of the diamine conjugate (*L1*)

The diamine conjugate **L1** was prepared by a four-step procedure from **1** employing 2,3-diaminopropionic acid (as a chelating group for platinum) linked to one carboxylate of **1** (α or γ) through ethylenediamine as a spacer (Scheme 1, where for sake of simplicity the spacer is represented linked to the γ carboxylate only). Aware of the previous problems connected to DCC, we decided to explore the coupling reaction between folic acid and mono (Boc)-ethylenediamine in the presence of a non-carbodiimide coupling agent. The first set of conditions tested was mixed anhydride formation with isobutyl chloroformate (IBCF) [17] or pivaloyl chloride [18] but this approach came to naught. Other protocols via uronium or phosphonium salt of a non-nucleophilic anion (PF_6^-) such as *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-bis(tetramethylene)uronium hexafluorophosphate (HBPyU) [19], (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) [20], (benzotriazol-1-yloxy)tris(dimethylamino) phosphonium hexafluorophosphate (BOP, Castro's reagent) [21] and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) [22] were examined in the attempt to limit by-product formation, but in all instances, either low yields were obtained or isolation problems occurred. Attempts were made to use other condensing agents (*e.g.*, diphenylphosphoryl azide (DPPA) [23] and diethyl cyanophosphonate (DECP) [24]), but none proved successful. After a concerted literature survey to look for potential solutions, one of the few reagents we found to be right for the purpose was 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT). CDMT (also known as Kaminski's reagent) [25] has been shown to be a versatile reagent in terms of stability, mild reaction conditions and cost. Gratifyingly, it was found that a DMF solution of **1** reacts smoothly (0° → r.t./20 h) with mono(Boc)-ethylenediamine upon addition of CDMT and *N*-methylmorpholine (NMM). The characterisation (^{13}C NMR) indicated that no diadducts between **1** and mono(Boc)-ethylenediamine were formed, whereas there were only monoadducts either on α or γ carboxylic group. The resulting Boc-amide **2** (86%) was *N*-(Boc)-deprotected (neat TFA, r.t., 1 h) and used directly in the coupling reaction with 2,3-di(Boc)-2,3-diaminopropionic acid. The successful iteration of Kaminski's protocol in DMF (0° → r.t./20 h) and subsequent Boc-deprotection of **3** in neat TFA at r.t. for 3 h led to desired ligand **L1** (as trifluoroacetate) (48% over three steps). The complex *cis*-(**L1**)$PtCl_2$ was obtained in good yield by reaction of $K_2[PtCl_4]$ and **L1** according to Miller's procedure for platinum coordination [26]. Thus, $K_2[PtCl_4]$ was added to an aqueous solution of **L1** (as trifluoroacetate) at about 60 °C. During the reaction, the pH was kept at 6.0 by the dropwise addition of 0.1N KOH. The pH value is important at this stage of the reaction: it must be high enough to deprotonate the diamine ligand, thus making it suitable for platinum coordination, but not so high as to cause the formation of inactive hydroxo-Pt species. Spectral evidence (multinuclear NMR, namely ^{1}H, ^{13}C and ^{195}Pt NMR) strongly supports the structures assigned to **L1** and (**L1**)$PtCl_2$. The characterisation was confirmed with thermogravimetric analysis (TGA) on the solid sample and inductively coupled plasma mass spectrometry



Scheme 1. Sketch of the synthesis of *cis*-[(2-(4-((2-amino-4-hydroxy-pteridin-6-ylmethyl)-amino)-benzoylamino)-4-(2-(2,3-bis-*tert*-butoxycarbonylamino-propionylamino)-ethylcarbamoyl)-butyric acid) dichloride platinum(II)], (**L1**)$PtCl_2$; Boc(en) = mono(Boc)-ethylenediamine, CDMT = 2-chloro-4,6-dimethoxy-1,3,5-triazine, DMF = dimethylformamide, NMM = *N*-methylmorpholine, TFA = trifluoroacetic acid.

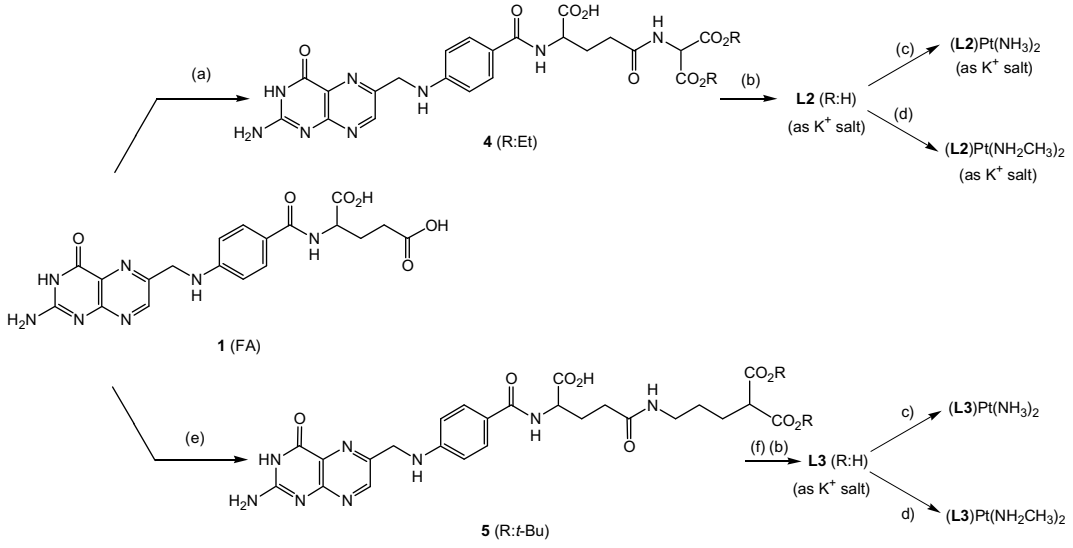
(ICP-MS) on the solution of the mineralised sample. In the TGA experiments, after heating of the sample in air atmosphere from r.t. to 1000 °C, the organic moiety was completely burnt and the platinum recovered as pure metal together with K_2O [27]. The ICP-MS analysis confirmed the Pt content in the mineralized sample.

Unfortunately, (**L1**)$PtCl_2$ showed low solubility in DMSO and was completely insoluble in water, thereby precluding any biochemical and biological test.

3.3. Synthesis of the dicarboxylic conjugates

Also in the case of the synthesis of the dicarboxylic conjugates, the use of carbodiimide-based coupling agents such as DCC [10a,14], and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC) [28], as well as diphenylphosphoryl azide (DPPA) [23] and diethyl cyanophosphonate (DECP) [24] resulted in a number of by-products and poor yield. Once again CDMT/NMM proved to be efficient as coupling agent for the functionalization of **1**. Therefore, the conditions chosen for coupling in the synthesis of folic acid-based dicarboxylic ligands **L2** and **L3** were those developed by Kaminski [25]. Pleasingly, we found the same reactivity pattern. For example, when **1** was reacted with diethyl aminomalonate hydrochloride with CTDM in DMF in the presence of NMM (0° → r.t.; 20 h), the amide **4** was obtained as the virtually exclusive product (41%) along with unreacted **1**. The same behaviour was observed when **1** reacted with di(*t*-butyl)-2(3-aminopropyl)malonate [8] under similar conditions resulting in the formation of **5**. The characterisation (^{13}C NMR) indicated that no diadducts between **1** and R-malonate [R = 2-amino; 2-(3-aminopropyl)] were formed, whereas there were only monoadducts either on α or γ carboxylic group. The products were sufficiently pure (NMR) for further reactions. Thus, ligand **L2** (as K^+ salt) was prepared in quantitative yield by exposure to KOH aqueous solutions at r.t. whereas conversion of **5** to **L3** was accomplished under standard conditions (neat TFA, r.t.), followed by exposure to KOH aqueous solutions at r.t. (Scheme 2, where for sake of simplicity the spacer is represented linked to the γ carboxylate only). The complexation of **L2** and **L3** was carried out according to Dhara's protocol [29] because it is rapid, easy and efficient. Thus, a solution of $K_2[PtI_4]$ was reacted with NH_3 or NH_2CH_3 to produce *cis*-$[(NH_2R)PtI_2$ (R = H or CH_3). The following replacement of iodide ions with water by means of Ag_2SO_4 or $AgNO_3$, yielded the corresponding diaqua-complex *cis*-$[(NH_2R)Pt(H_2O)_2]^{2+}$. The treatment of the **L2** and **L3** (as K^+ salts) with a solution of *cis*-$[(NH_2R)Pt(H_2O)_2]^{2+}$ yielded the final complexes (Scheme 2). We chose to synthesise these Pt-complexes using two different amines: NH_3 in order to get cisplatin-like complexes, namely (**L2**)$Pt(NH_3)_2$ and (**L3**)$Pt(NH_3)_2$, and NH_2CH_3 in order to improve the water solubility of the resulting compounds, namely (**L2**)$Pt(NH_2CH_3)_2$ and (**L3**)$Pt(NH_2CH_3)_2$, even if the methylation is known to be detrimental for the cytotoxic activity [30].

The ligands **L2** and **L3** are soluble in some polar organic solvents (*i.e.*, DMF and DMSO) and in aqueous alkaline solutions and fully characterised by multinuclear NMR spectroscopy. Unfortunately, the corresponding Pt complexes showed solubility traits similar to the other folic acid-based (**L1**)$PtCl_2$ complex (*vide supra*). This precluded any multinuclear NMR analyses whereas both TGA and ICP-MS confirmed the proposed molecular formulas for these complexes.



a) diethyl aminomalonate hydrochloride, CDMT, NMM, DMF, from 0°C to r.t.; b) KOH, H_2O, r.t.; c) *cis*-$[(NH_3)_2Pt(H_2O)_2]^{2+}$, H_2O; d) *cis*-$[(NH_2CH_3)_2Pt(H_2O)_2]^{2+}$, H_2O; e) di(*t*-butyl)-2-(3-aminopropyl)malonate, CDMT, NMM, DMF, from 0°C to r.t.; f) TFA

Scheme 2. Sketch of the synthesis of folic acid derivatives and their corresponding Pt-complexes. CDMT = 2-chloro-4,6-dimethoxy-1,3,5-triazine, DMF = dimethylformamide, NMM = *N*-methylmorpholine, TFA = trifluoroacetic acid.

In order to further corroborate the structural assignments, Raman and IR spectra were performed. Raman spectroscopy is useful to evaluate the shift of the stretching bands of carboxylic groups from free ligands to complexes and the presence of ν(Pt–X) bands of the complexes. As reported in the literature [31], ν(Pt–N) bands are in the range 600–400 cm^{-1} while ν(Pt–O) between 500 and 400 cm^{-1}. In the free ligands the ν(C=O) bands are between 1800 and 1650 cm^{-1} [32], whereas in the corresponding complexes ν(C=O) bands are in the 1750–1560 cm^{-1} region. Unfortunately, all the complexes of ligands **L2** and **L3** were fluorescent and the diagnostic bands in the Raman spectra were hidden by this fluorescence. Therefore, we could not evaluate the coordination of the ligands to platinum by means of Raman spectroscopy. On the contrary, the IR spectroscopy was useful to evaluate the shift of ν(C=O) of carboxylic groups from free ligands to complexes, even though the spectra were highly crowded due to the various functional groups of the molecules. In particular, the spectrum of free malonic acid itself shows a characteristic strong band at 1725 cm^{-1}, that moves in the spectrum of the corresponding *cis*-[$(NH_3)_2$Pt(malonate)] complex to 1625 cm^{-1}. The same situation holds in the comparison of the spectra of **L2** and **L3** with those of the corresponding Pt-complexes. Ligands **L2** and **L3** show a strong band at around 1729 cm^{-1} that shifts to 1638 cm^{-1} in the complexes. These shifts of the vibrational bands, together with the TGA and ICP-MS data, indicate that complexes (**L2,3**)Pt(NH_2R) (R = H or CH_3) have been successfully synthesized and purified but once again their low solubility precluded any biochemical and biological evaluations.

Further studies will be focused upon designing a new series of Pt compounds linked to folic acid-based vectors made more water-soluble by insertion of polar functionalities.

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