Synthesis and cytotoxicity of dinuclear complexes containing ruthenium(II) bipyridyl units linked by a bis(pyridylimine) ligand†‡

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Enantiopure dinuclear ruthenium polypyridyl complexes of the form $[Ru_2(LL)_4\mathbf{L^1}](PF_6)_4$ (LL = 2,2′-bipyridine (bpy) or 1,10-phenanthroline (phen); $\mathbf{L^1} = C_{25}H_{20}N_4$ a bis(pyridylimine) ligand containing a diphenylmethane spacer) have been synthesized using the chiral building blocks *cis*-$[Ru(bpy)_2(py)_2]^{2+}$ and *cis*-$[Ru(phen)_2(py)_2]^{2+}$. These dinuclear ruthenium complexes have been characterised using NMR, mass spectrometry, UV-visible absorbance, circular dichroism and linear dichroism. The compounds exhibit good photo and thermal stability. The extinction coefficient for the bpy complex at 478 nm is $\varepsilon_{478} = 15\,700$ mol^{-1} cm^{-1} dm^3 and for the phen complex is $\varepsilon_{478} = 24\,900$ mol^{-1} cm^{-1} dm^3. Both complexes have their longest wavelength (metal to ligand charge transfer) transition predominantly *x*/*y* (short axis)-polarised while the transitions at shorter wavelength are a mixture of *x*/*y* and *z*-polarisations, similar to both the copper helicate and iron triple helicate studied previously. Cytotoxicity studies reveal that the compounds are dramatically less active against cancer cell lines than the recently reported supramolecular cylinders prepared from the same bis(pyridylimine) ligand.

Introduction

Complexes of transition metal cations play an important role in combating cancers. Indeed, four platinum(II) complexes (cisplatin, carboplatin, nedaplatin and oxaliplatin) are routinely used in the clinic to treat patients with a range of cancers, notably testicular and ovarian.[1] Almost all clinical regimens of combination therapy used against aggressive cancers employ one of these drugs which are broad cytotoxics that act by binding through coordination bonds to the purine bases of DNA. Despite their clinical value, these platinum agents are far from ideal and challenges include alleviating side effects, widening their spectrum of activity to treat other cancers, and overcoming acquired drug resistance. A key to addressing these challenges is to design agents that act on cellular components in quite different ways to the platinum drugs and this has led scientists to explore a variety of other designs.

One promising approach has centred around ruthenium complexes some of which have shown promising anti-tumour activity[2–4] and two of which, NAMI-A[3] and KP1019,[4] are currently in clinical trials for the treatment of metastatic and colorectal cancers respectively. The precise modes of action and biomolecular targets of these ruthenium drugs are less well established than in the case of cisplatin. Ruthenium itself is among the most studied of the transition metal ions, in large part because of the interesting photophysical and redox properties of its complexes with polypyridyl ligands. There has been great interest in such ruthenium(II) polypyridyl complexes as potential biologically active agents and as building blocks in supramolecular devices.[1–4] Derivatives of the tris-chelate complexes $[Ru(bpy)_3]^{2+}$ and $[Ru(phen)_3]^{2+}$ (2,2′-bipyridine (bpy) or 1,10-phenanthroline (phen)) have also been explored as luminescent structural probes for DNA.[6] Of particular relevance is a series of dinuclear complexes designed by Lincoln and Nordén which possess two $[Ru(phen)_2dppz]^{2+}$ units linked together using a bridging ligand. This creates bis-intercalating agents which thread through the DNA.[7] Alongside their unique mode of binding to DNA, these compounds also demonstrate activity against cancer-cell lines.[8] By contrast, the simple tris-chelate $[Ru(bpy)_3]^{2+}$ (a DNA groove binder) is reported to be inactive.[9]

In a different approach, we have explored the DNA-binding of metallo-supramolecular cylinders that are a similar size and shape to zinc fingers and other α-helical motifs found in certain DNA-recognition proteins.[10–12] These tetracationic cylinders (such as $[M_2(\mathbf{L^1})_3]^{4+}$, Fig. 1 and 2) are assembled from bis(pyridylimine) ligands containing, for example, a diphenylmethane spacer.[13] They are comprised of three such ligand strands wrapped in a helical fashion about two dicationic metal centres and, in contrast to clinical platinum metallo-drugs, they bind non-covalently to DNA. The cylinders can not only bind strongly in the major groove

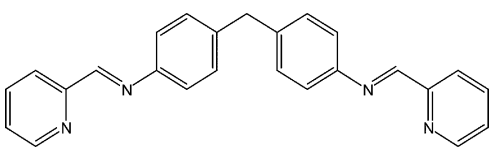

Fig. 1 Bis(pyridylimine) ligand $\mathbf{L^1}$.

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† The HTML version of this article has been enhanced with colour images.
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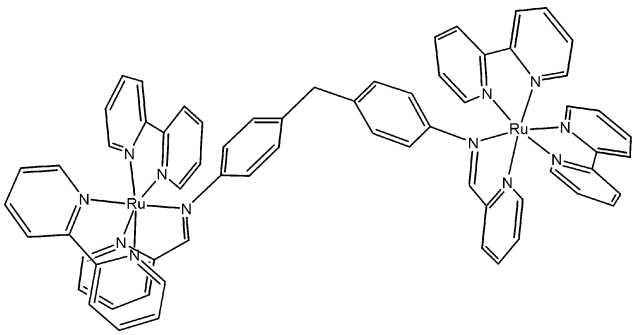

Fig. 2 Example of the target structures of type $[Ru_2(bpy)_4\mathbf{L^1}](PF_6)_4$.

of DNA, inducing dramatic and unprecedented intra-molecular DNA coiling in natural polymeric DNAs,[10] but can also bind at the heart of Y-shaped DNA junctions,[12] an unparalleled and hitherto unexpected mode of DNA recognition. Excitingly we have shown that these cylinders possess similar levels of potency against cancer cell lines as the platinum drugs despite their completely different mode of binding.[14]

NMR and X-ray structural studies indicate that the molecular surface of these cylinders are a key to their unique properties, with the diphenylmethane spacer units of the ligand playing a significant role in the recognition of DNA junctions.[12] To deepen our knowledge of the structure–activity relationships in these systems, we explore herein the synthesis and cellular activity of alternate but related structures in which a single bis(pyridylimine) ligand is used to link two ruthenium bis(diimine) centres. The resulting arrays will be similar to the cylinders in so far as they are dinuclear, tetracationic and linked by a diphenylmethane spacer. However, they will be more flexible and will not contain the large polyaromatic surface at their centre which facilitates junction recognition.

The work builds upon our previous studies on the design and photophysics of mononuclear mixed-ligand pyridylimine/bipyridine complexes.[15] Two previous reports of dinuclear ruthenium bis(diimine) complexes linked by pyridylimine ligands have appeared,[16,17] one of which contains the racemic version of $[Ru_2(bpy)_4\mathbf{L^1}]^{4+}$ (prepared only as a mixture of diastereoisomers).[16] The activity of such compounds against cancer cell lines has not previously been explored. Indeed studies of the cellular activity of dinuclear ruthenium tris(diimine) complexes are rare.[8]

Results and discussion

The basic target structure is illustrated in Fig. 2. The introduction of tris-bidentate metal centres brings issues of chirality, which if not controlled lead to mixtures of diastereoisomers. In order to prepare complexes with predetermined chirality, enantiopure chiral building blocks were used as starting materials: *cis*-$[Ru(phen)_2(py)_2]^{2+}$ and *cis*-$[Ru(bpy)_2(py)_2]^{2+}$. These chiral precursors can be readily separated into their enantiomers and then undergo substitution reactions with bidentate ligands with retention of configuration.[5]

Synthesis and characterisation

$\mathbf{L^1}$ was prepared as we have previously described.[13] In the first instance, racemic ruthenium bis(diimine) starting materials *cis*-$[Ru(LL)_2(X)_2]$ (X = Cl, LL = phen or bpy) were prepared using literature procedures.[22–26] Reaction of the ligand with an excess of the racemic *cis*-$[Ru(LL)_2Cl_2]$ in ethylene glycol afforded the target compounds as diastereomeric mixtures. Attempts to resolve the isomers from the diastereomeric mixtures by chromatographic techniques were unsuccessful, so the enantiopure starting material approach was used to provide enantiopure products. The enantiopure *cis*-$[Ru(LL)_2(py)_2]^{2+}$ starting materials were prepared and used to produce the enantiopure Δ,Δ and Λ,Λ isomers. The compounds were isolated as the hexafluorophosphate salts. The chloride salts of the compounds were obtained by adding acetone solutions of tetra-*n*-butylammonium chloride to acetone solutions of the hexafluorophosphate compounds.

The dinuclear compounds show peaks (with the correct isotopic distributions) in their electrospray mass spectra corresponding to $\{Ru_2(LL)_4\mathbf{L^1}(X)_3\}^+$, $\{Ru_2(LL)_4\mathbf{L^1}(X)_2\}^{2+}$ and $\{Ru_2(LL)_4\mathbf{L^1}(X)\}^{3+}$. Microanalytical data are consistent with their proposed formulations. The compounds prepared from the racemic starting materials are a mixture of the *meso*-(Δ,Λ) and *rac*-(Δ,Δ, and Λ,Λ) isomers as is evident in the NMR spectra. The two diastereoisomeric molecules have very similar NMR spectra, but a doubling of many of the signals can just be detected. This is most evident in the spectra of the bpy compound in d_6-acetone or d_3-acetonitrile in which the phenyl resonances each appear as two overlapping doublets. While the *meso*- and *rac*-peaks are insufficiently separated for accurate integration, they do appear to be present in an approximately 1 : 1 ratio, consistent with little or no enantioselection taking place during the addition of the second metal centre (as would be expected). By way of contrast, the compounds prepared from the enantiopure starting materials show single sets of peaks; the absence of *meso*-peaks for these compounds, taken with the CD results presented below, serves to confirm their enantiopurity (at least within the limits of NMR detection).

UV/Visible absorption spectroscopy of the metal complexes

The UV/Visible absorbance spectra of aqueous solutions of $[Ru_2(bpy)_4\mathbf{L^1}]^{4+}$ and $[Ru_2(phen)_4\mathbf{L^1}]^{4+}$ species are shown in Fig. 3. The complexes show a broad MLCT (metal ligand charge transfer) envelope between 400 and 550 nm with a structure that indicates (at least) two overlapping bands. A red shift of the ^{1}MLCT absorption band in Ru(II) complexes on replacing pyridine ligands with imines has been previously reported consistent with a lower energy π* level than with the bipyridine ligand.[15] Additional absorption bands below 300 nm are characteristic of in-ligand transitions. The spectra are in accord with those of mononuclear mixed bipyridine-pyridylimine complexes.[15] The extinction coefficients for the compounds are given in Table 1.

Table 1 Extinction co-efficient (ε) values of the metal complexes in aqueous solutions

Ruthenium complex	In-ligand ε/mol^{-1} cm^{-1} dm^3	MLCT ε/mol^{-1} cm^{-1} dm^3
Rac-$[Ru_2(bpy)_4L]^{4+}$	$\varepsilon_{285} = 89\,900$	$\varepsilon_{442} = 15\,900$ $\varepsilon_{478} = 15\,700$
Rac-$[Ru_2(phen)_4L]^{4+}$	$\varepsilon_{263} = 186\,400$	$\varepsilon_{427} = 28\,900$ $\varepsilon_{478} = 24\,900$

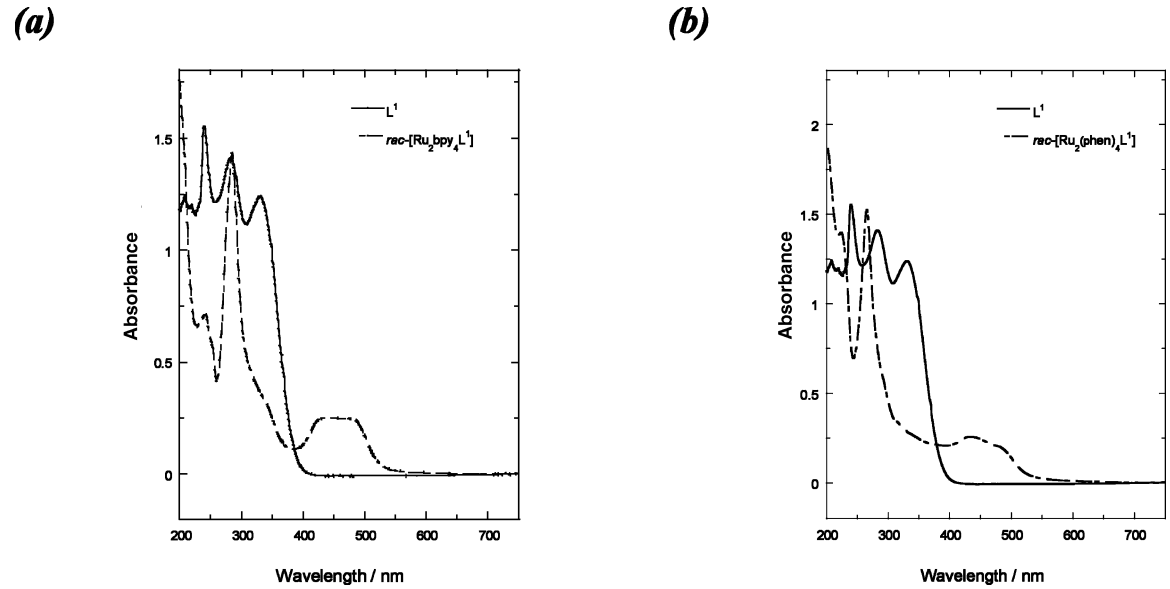

Fig. 3 UV-visible absorbance spectra of aqueous solutions (20 μM, 1 cm path length) of (a) $[Ru_2(bpy)_4\mathbf{L^1}]^{4+}$ and (b) $[Ru_2(phen)_4\mathbf{L^1}]^{4+}$. The spectra shown are for the *meso*- and *rac*-mixtures prepared from the racemic starting materials. The UV-visible spectrum of $\mathbf{L^1}$ is also shown for comparison.

CD spectroscopy of the metal complexes

The CD spectra of aqueous solutions of the enantiomers of $[Ru_2(bpy)_4\mathbf{L^1}]^{4+}$ and $[Ru_2(phen)_4\mathbf{L^1}]^{4+}$ are shown in Fig. 4. The spectra for unresolved $[Ru_2(bpy)_4\mathbf{L^1}]^{4+}$ and $[Ru_2(phen)_4\mathbf{L^1}]^{4+}$ are flat lines of zero magnitude confirming the presence of an equal ratio of the two *rac*-enantiomers in the mixture as expected. The enantiomers of the metal complexes have a characteristic CD pattern below 600 nm. The CD spectra of the enantiomeric pairs of each complex show equal magnitude and opposite signed CD signals when scaled to the same normal absorbance maximum at 285 nm thus leading us to conclude that the compounds are close to 100% enantiomerically pure since it is unlikely that exactly the same degree of purity would result from two separate experiments unless it is 100% (or 0%). (This is consistent with previous literature confirming that the enantiopure starting materials are added to the ligand with retention of configuration.)[18]

Film LD

Film absorbance and film LD spectra of the racemic complexes were measured to qualitatively assign their transition moment polarisations as follows. A 10% (w/v) low molecular weight aqueous solution of polyvinyl alcohol was prepared, heated to 100 °C and allowed to cool. A 0.2 mL saturated aqueous solution of the chosen metal complex was added to 4.8 mL of cooled

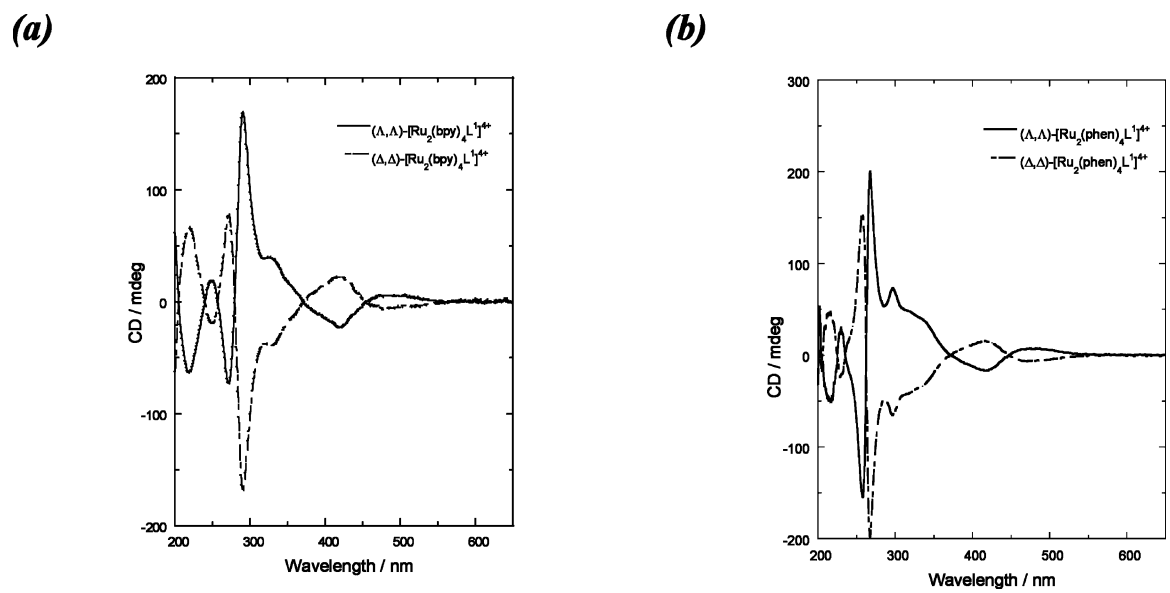

Fig. 4 CD spectra of aqueous solutions of (a) (Δ,Δ) and (Λ,Λ)-$[Ru_2(bpy)_4\mathbf{L^1}]^{4+}$ (15 μM, 1 cm path length) and (b) (Δ,Δ) and (Λ,Λ)-$[Ru_2(phen)_4\mathbf{L^1}]^{4+}$ (15 μM, 1 cm path length).[27,28]

PVA solution. The solution was mixed and then poured onto a glass plate and allowed to dry in a dust free environment (at room temp for 2–3 d). A blank PVA film was also prepared in which water replaced the metal complex solution. Both the PVA films were carefully removed from the glass slides and placed in a mechanical stretcher and stretched under heat. LD and UV/Visible absorbance measurements were taken of both films before stretching and with increasing stretching until the spectra remained constant. The PVA blank was subtracted from the metal complex spectra.

Component spectra were calculated following published methods[19,20] which are based on that of Thulstrup.[21] It is assumed that the ruthenium bimetallo complexes were rod-like and thus adopted uniaxial orientation in the film. One then assumes either that the largest positive reduced LD (LD^r = LD/Absorbance) is purely *z*-polarised or the largest negative signal is purely *x*/*y* polarised. The validity of that assumption can be tested by incrementing the orientation parameters and inspecting the resulting component spectra visually. In this case we have chosen to use the largest positive LD^r signal and assume it is purely *z*-polarised. The resulting spectra and the calculated component spectra are shown in Fig. 5. The longest wavelength (MLCT) transition is predominantly *x*/*y* (short axis)-polarised while the transitions at shorter wavelength are a mixture of *x*/*y* and *z*-polarisations, similar to both the copper helicate and the iron triple helicate studied previously.[10,11]

Stability

Photostability and thermal stability experiments were carried out by preparing 20 μM solutions of (+)-$[Ru_2(bpy)_4\mathbf{L^1}]^{4+}$ and of (+)-$[Fe_2\mathbf{L^1}_3]^{4+}$ for comparison purposes.[13] The solutions of the metal complexes were placed in the dark (wrapped in aluminium foil and placed in a drawer at ambient temperature), and in the light (left on a laboratory bench at ambient temperature) to explore the photostability. For thermal stability the solutions of the metal complexes were placed in ovens at 37 °C and 60 °C, and in a refrigerator at 4 °C (hence these experiments are under 'dark' conditions). UV/Visible absorbance spectra and CD spectra were recorded over a period of 9 months.

The UV/Visible and CD spectra of the ruthenium complex stored at room temperature in dark and light and in the dark at 4 °C, 37 °C and 60 °C (see Fig. 6 and ESI‡) show a negligible loss of signal intensity in the MLCT and in-ligand region of the spectra. These results show that the ruthenium complex is chemically and configurationally stable in both dark and light conditions over a period of nine months except at 60 °C. The di-iron triple helicate,[13] (+)-$[Fe_2\mathbf{L^1}_3]^{4+}$, is less stable. Although it is sufficiently stable for periods of days at room temperature for DNA binding and biological activity to be assessed, over prolonged periods, in both the dark and light conditions, the UV/Visible and CD intensity of the iron complex decreases significantly in both the MLCT and in-ligand region.

Cytotoxicity

The cytotoxicity of the hexafluorophosphate salts of the compounds has been tested against HBL100 (human breast cancer) and SKOV-3 (ovarian carcinoma) cell lines. The solubility of these fairly high molecular weight compounds is such that the highest concentration that could be used (while only exposing the cells to a maximum of 1% dmso) was 112 μM for the $[Ru_2(bpy)_4\mathbf{L^1}](PF_6)_4$ compounds and 80 μM for the $[Ru_2(phen)_4\mathbf{L^1}](PF_6)_4$ compounds.

These cytotoxicity experiments showed that the compounds hardly inhibit the cell proliferation. The solubility limitations coupled with the low activity of the compounds, prevented the full concentration-dependence curve for inhibition of cell growth from being completed. Hence absolute IC_{50} could not be obtained, although estimates can be made from the experimental data obtained.

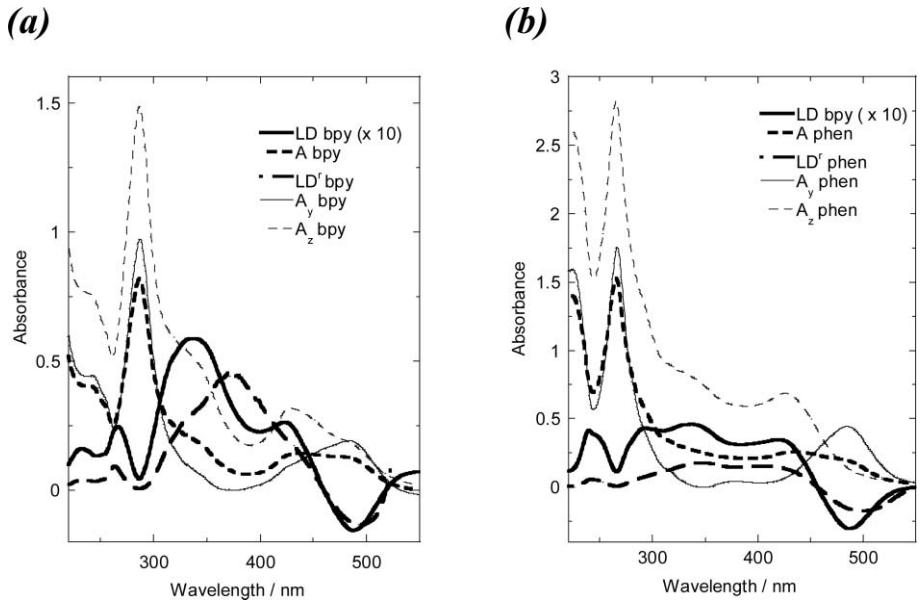

Fig. 5 Film LD, UV and LD^r spectra of PVA films of (a) $[Ru_2(bpy)_4\mathbf{L^1}]^{4+}$ and (b) $[Ru_2(phen)_4\mathbf{L^1}]^{4+}$. Also shown are the component polarised spectra for *z* component absorbance and *y* component absorbance assuming uncorrected orientation parameters could be used.

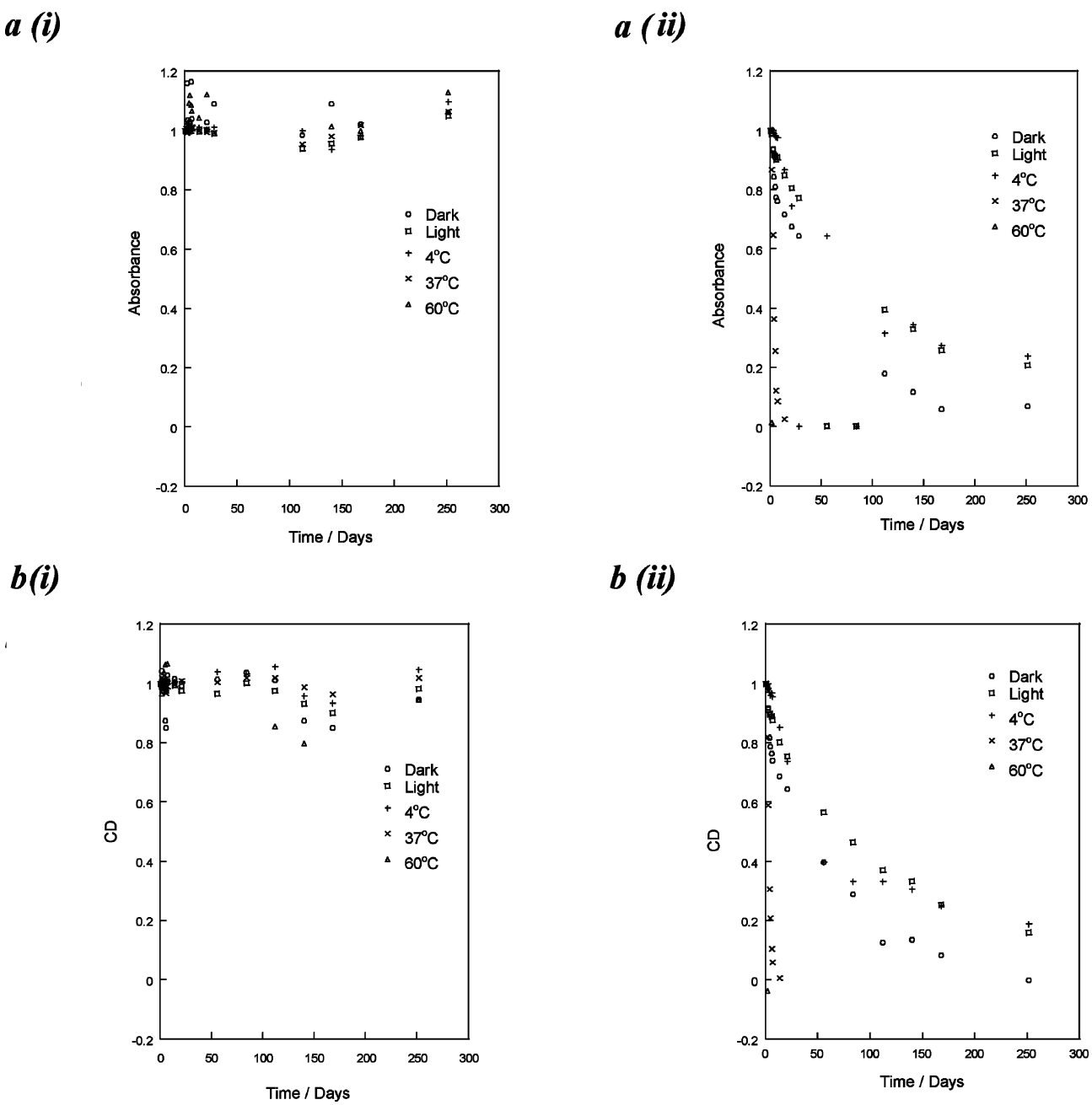

Fig. 6 UV/Visible absorbance intensity plots of a(i) (+)-$[Ru_2(bpy)_4\mathbf{L^1}]^{4+}$ at 478 nm and a(ii) (+)-$[Fe_2L_3{}^1]^{4+}$ at 510 nm. CD intensity plots of b(i) (+)-$[Ru_2(bpy)_4\mathbf{L^1}]^{4+}$ at 420 nm and b(ii) (+)-$[Fe_2\mathbf{L^1}_3]^{4+}$ at 512 nm. Solutions were stored in darkness, light, 4 °C, 37 °C and 60 °C. The spectra were collected in 1 cm (light, 4 °C, 37 °C) and 0.5 cm (dark, 60 °C) path length cuvettes. The data are normalised to 1 at time zero.

In HBL100 cells (human breast cancer), the highest concentration used (112 μM) of the three $[Ru_2(bpy)_4\mathbf{L^1}](PF_6)_4$ compounds (the *meso*- and *rac*-mixture; the ΛΛ isomer; the ΔΔ isomer) showed 50–60% cell growth. No significant differences between the mixture and the enantio-pure complexes was observed. The highest concentration used (80 μM) of the three $[Ru_2(phen)_4\mathbf{L^1}](PF_6)_4$ compounds inhibited cell growth in the HBL100 cells by only around 50%. It is thus apparent that the IC_{50} values for these compounds would lie at around 80–100 μM; values that would usually be classed as very low activity. Cisplatin shows an IC_{50} value of 5 μM in this cell line.

In SKOV-3 cells (ovarian carcinoma) the highest concentration (112 μM) of the three $[Ru_2(phen)_4\mathbf{L^1}](PF_6)_4$ compounds only inhibited cell growth by 30%. The highest conc. (80 μM) of the *meso*- and *rac*-mixture of $[Ru_2(phen)_4\mathbf{L^1}](PF_6)_4$ inhibited cell growth with 40%, whereas the ΛΛ and ΔΔ $[\{Ru(phen)_2\}_2L](PF_6)_4$ only inhibited cell growth with 25%. It is apparent that in this cell line the IC_{50} values for these compounds would lie above 100 μM. In the case of the phenanthroline compound the slight difference between the effects of the compounds prepared from racemic and enantiomeric pure starting materials may reflect a slightly enhanced activity for the *meso* isomer.

Conclusions

A series of the desired $[Ru_2(LL)_4\mathbf{L^1}](PF_6)_4$ compounds have been successfully prepared, including the enantiopure ΛΛ and ΔΔ isomers. The compounds exhibit good photo and thermal stability in solution. Cytotoxicity studies reveal that the compounds are considerably less active against cancer cell lines than the recently reported supramolecular cylinders prepared from the same bis(pyridylimine) ligand.[14] This result emphasises the importance of the precise structure of those cylinders in their activity and confirm that it is not simply individual components (such as the individual ligand structure or the tetracationic charge) of the cylinder that give rise to the activity. This is consistent with the precise and unusual mode(s) of interaction with DNA being crucial to the activity. The results are also revealing in the context of Lincoln and Nordén's threading bis-intercalators.[8] Those dinuclear ruthenium complexes, which also possess a unique and unusual DNA binding mode, exhibit good activity in cell lines while the dinuclear ruthenium compounds herein do not. Again this points to cellular activity being linked to the potential for unique interactions with biomolecular targets rather than being a simple effect of the charge and size of a class of molecule. Studies to probe the DNA interactions of these new compounds in detail are ongoing.

Experimental

^{1}H NMR studies were carried out on a DPX 300 and a DPX 400 MHz Bruker spectrometer operated in Fourier transform mode. Complete assignment of the signals in the ^{1}H NMR spectrum was done using 2D NMR COSY (through band correlation) and NOESY experiments (through space correlation). Infra-red spectra were recorded on a Perkin Elmer FTIR spectrometer on the powdered form of the samples. LSI-MS were recorded on a Micromass Autospec spectrometer, and electrospray mass spectra (ESI-MS) were recorded on a Micromass Quatro II (low resolution triple quadropole mass spectrometer) instrument at the EPSRC National Mass Spectrometry Centre, University of Wales, Swansea or on a Bruker esquire 2000 electrospray mass spectrometer at the University of Warwick. Elemental analyses were performed by Warwick Analytical Services Ltd. UV/Visible absorbance measurements were carried out using a Jasco V-550 spectrophotometer. CD spectra were collected on a Jasco J-715 spectropolarimeter. Starting materials and reagents were purchased from Sigma-Aldrich or Fluka and used without further purification.

$\mathbf{L^1}$ and the complex (+)-$[Fe_2\mathbf{L^1}_3]^{4+}$ were prepared according to our previously described proceedures.[13] *Cis*-$[Ru(bpy)_2Cl_2]\cdot 2H_2O$ was prepared following the literature method of Meyer.[22] Racemic $[Ru_2(bpy)_2(py)_2](Cl_2)$, (py = pyridine) was prepared according to the procedure of Morgan and Wang.[23] The Λ and Δ isomers of $[Ru_2(bpy)_2(py)_2]^{2+}(Cl_2)$ were isolated by the addition of an aqueous solution of *O*,*O*′-dibenzoyl-D-tartrate (0.25 M) to a solution of racemic $[Ru_2(bpy)_2(py)_2]^{2+}(Cl_2)$ in water following the method of Von Zelewsky.[18] Upon slow evaporation, red crystals of the pure Δ salt were formed and were collected by filtration. The pure Λ salt was prepared by the same method but using *O*,*O*′-dibenzoyl-L-tartrate (0.25 M). Circular dichroism (CD) spectra were recorded to confirm its enantiomeric composition with reference to literature data.[23]

[6] For the bis(phenanthroline) complex, the precursor *cis*-$[Ru(phen)_2Cl_2]^{2+}$, was prepared,[24] added to aqueous pyridine, and then treated with diethyl ether to afford yellow *cis*-$[Ru(phen)_2(py)_2]Cl_2$.[25] This cation was resolved into its optical isomers, Δ and Λ, by precipitation with sodium arsenyl-D-tartrate (0.80 M) and sodium arsenyl-L-tartrate (0.80 M) respectively.[26]

$[Ru_2(bpy)_4\mathbf{L^1}](PF_6)_4$

$[Ru_2(bpy)_4\mathbf{L^1}](PF_6)_4$ was prepared by dissolving *cis*-$[Ru(bpy)_2Cl_2]$ (0.15 g, 0.31 mmol) and $\mathbf{L^1}$ (0.034 g, 0.090 mmol) in ethylene glycol (8 mL). The solution was heated at 120 °C for 6 h in the dark. After cooling to room temperature the solution was diluted with MeOH (6 mL). A saturated methanolic solution of NH_4PF_6 was added drop wise until no more precipitate formed. The precipitate was filtered, dried under reduced vacuum and recrystallised from an acetonitrile–diethyl ether mixture. The final brown precipitate was filtered, washed with diethyl ether (2 × 25 mL) and dried under reduced vacuum (0.12 g, 72% yield).

^{1}H NMR (400 MHz, d_6-acetone): δ 9.01 (s, 1H, H_5), 8.54 (m, 3H, H_{14}, H_{22}, H_{26}), 8.32 (d, 1H, J = 7.7 Hz, H_4), 8.12 (m, 5H, H_3, H_{13}, H_{21}, H_{23}, H_{25}), 7.90 (d, 1H, J = 8.2 Hz, H_{18}), 7.80 (d, 1H, J = 5.5 Hz, H_{11}), 7.75 (d, 1H, J = 5.4 Hz, H_{19}), 7.69 (d, 1H, J = 5.7 Hz, H_1), 7.64 (m, 1H, H_{17}), 7.56 (m, 3H, H_2, H_{15}, H_{24}), 7.48 (t, 1H, J = 6.0, H_{12}), 7.39 (t, 1H, J = 6.2 Hz, H_{20}), 7.17 (t, 1H, J = 6.1, H_{16}), 6.69 (dd, 2H, J = 2.3 Hz, J = 8.4 Hz, H_a), 6.46 (d, 2H, J = 8.2 Hz, H_b), 3.62 (s, 1H, CH_2). LSI-MS: *m*/*z* 1639 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)_3\}^+$, 1494 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)_2\}^+$, 1349 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)\}^+$, 747 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)_2\}^{2+}$. ESI: *m*/*z* 1639 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)_3\}^+$, 747 $\{Ru_2\ (bpy)_4\mathbf{L^1}(PF_6)_2\}^{2+}$, 449 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)\}^{3+}$. CHN: Calc. for $C_{65}H_{52}N_{12}Ru_2P_4F_{24}\cdot 0.5CH_3CN$: C, 43.95; H, 2.99; N, 9.71%. Found: C, 43.91; H, 3.23; N, 9.66%. Selected IR data (cm^{-1}): 3649(w), 3328(w), 1605(m), 1503(w), 1466(w), 1446(w), 1313(w), 1243(w), 1161(w), 1036(w), 833(s), 762(m), 730(w), 659(w).

(Δ,Δ)-$[Ru_2(bpy)_4\mathbf{L^1}](PF_6)_4$

(Δ,Δ)-$[Ru_2(bpy)_4\mathbf{L^1}](PF_6)_4$ was prepared by dissolving Δ-$[Ru(bpy)_2(py)_2]$(dibenzoyl tartrate)$_2$ (0.15 g, 0.263 mmol) and $\mathbf{L^1}$ (0.034 g, 0.090 mmol) in ethylene glycol (8 mL). The solution was heated at 120 °C for 6 h in darkness. After cooling to room temperature the solution was diluted with MeOH (6 mL). A saturated methanolic solution of NH_4PF_6 was added drop wise until no more precipitate formed. The precipitate was filtered, dried under reduced vacuum and recrystallised using an acetonitrile–diethyl ether mixture. The brown precipitate was washed with diethyl ether (2 × 25 mL) and dried under reduced vacuum (0.125 g, 78% yield).

^{1}H NMR (400 MHz, d_6-acetone, Fig. 7): δ 9.02 (s, 1H, H_5), 8.54 (m, 3H, H_{14}, H_{22}, H_{26}), 8.32 (d, 1H, J = 7.7 Hz, H_4), 8.12 (m, 5H, H_3, H_{13}, H_{21}, H_{23}, H_{25}), 7.90 (d, 1H, J = 8.2 Hz, H_{18}), 7.81 (d, 1H, J = 5.5 Hz, H_{11}), 7.75 (d, 1H, J = 5.5 Hz, H_{19}), 7.69 (d, 1H, J = 5.5 Hz, H_1), 7.65 (m, 1H, H_{17}), 7.56 (m, 3H, H_2, H_{15}, H_{24}), 7.48 (t, 1H, J = 6.6 Hz, H_{12}), 7.39 (t, 1H, J = 6.6 Hz, H_{20}), 7.17 (t, 1H, J = 6.6, H_{16}), 6.69 (t, 2H, J = 8.4 Hz, H_a), 6.46 (d, 2H, J = 8.3 Hz, H_b), 3.63 (s, 1H, CH_2). LSI-MS: *m*/*z* 1639 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)_3\}^+$, 1494 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)_2\}^+$, 1349 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)\}^+$, 747 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)_2\}^{2+}$. ESI: *m*/*z*

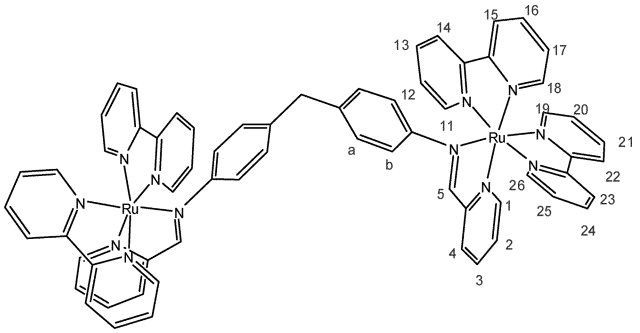

Fig. 7 $[Ru_2(bpy)_4\mathbf{L^1}](PF_6)_4$, protons numbered.

1639 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)_3\}^+$, 747 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)_2\}^{2+}$, 449 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)\}^{3+}$. CHN: Calc. for $C_{65}H_{52}N_{12}Ru_2P_4F_{24}$: C, 43.78; H, 2.94; N, 9.43%. Found: C, 43.64; H, 3.09; N, 9.11%. Selected IR data (cm^{-1}): 2357(w), 1501(w), 1467(w), 1427(w), 1184(w), 846(s), 798(s), 747(m), 730(m).

(Λ,Λ)-[Ru$_2$(bpy)$_4$L^1](PF$_6$)

(Λ,Λ)-$[Ru_2(bpy)_4\mathbf{L^1}](PF_6)$ was prepared from Λ-$[Ru(bpy)_2(py)_2]$-(dibenzoyl tartarte)$_2$ according to the procedure described for (Δ, Δ)-$[Ru_2(bpy)_4\mathbf{L^1}]^{4+}(PF_6)_4$. The precipitate was filtered, dried under reduced vacuum and recrystallised using an acetone/diethyl ether mixture. The brown precipitate was washed with diethyl ether (2 × 25 mL) and dried under reduced vacuum (0.117 g, 73% yield).

^{1}H NMR (400 MHz, d$_6$-acetone): δ 9.00 (s, 1H, H_5), 8.54 (m, 3H, H_{14}, H_{22}, H_{26}), 8.32 (d, 1H, J = 7.8 Hz, H_4), 8.11 (m, 5H, H_3, H_{13}, H_{21}, H_{23}, H_{25}), 7.90 (d, 1H, J = 8.1 Hz, H_{18}), 7.80 (d, 1H, J = 5.5 Hz, H_{11}), 7.75 (d, 1H, J = 5.3 Hz, H_{19}), 7.66 (m, 2H, H_1, H_{17}), 7.55 (m, 3H, H_2, H_{15}, H_{24}), 7.48 (t, 1H, J = 7.2 Hz, H_{12}), 7.39 (t, 1H, J = 6.6 Hz, H_{20}), 7.17 (t, 1H, J = 6.6 Hz, H_{16}), 6.69 (t, 2H, J = 8.3 Hz, H_a), 6.45 (d, 2H, J = 8.3 Hz, H_b), 3.62 (s, 1H, CH_2). LSI-MS: m/z 1639 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)_3\}^+$, 1493 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)_2\}^+$, 1349 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)\}^+$, 747 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)_2\}^{2+}$. ESI: m/z 1639 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)_3\}^+$, 747 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)_2\}^{2+}$, 449 $\{Ru_2(bpy)_4\mathbf{L^1}(PF_6)\}^{3+}$. CHN: Calc. for $C_{65}H_{52}N_{12}Ru_2P_4F_{24}\cdot(CH_3)_2CO$: C, 44.36; H, 3.18; N, 9.13%. Found: C, 44.58; H, 3.33; N, 8.94%. Selected IR data (cm^{-1}): 2356(w), 1502(w), 1466(w), 1427(w), 1183(w), 845(s), 799(s), 747(m), 731(m).

[Ru$_2$(phen)$_4$L^1](PF$_6$)$_4$

$[Ru_2(phen)_4\mathbf{L^1}](PF_6)_4$ was prepared by dissolving *cis*-$[Ru(phen)_2Cl_2]$ (0.081 g, 0.15 mmol) and **L^1** (0.019 g, 0.051 mmol) in ethylene glycol (4 mL). The solution was heated at 120 °C for 6 h in darkness. After cooling to room temperature the solution was diluted with MeOH (6 mL). A saturated methanolic solution of NH_4PF_6 was added drop wise until no more precipitate formed. The resulting precipitate was filtered, dried under reduced vacuum and recrystallised using an acetone–diethyl ether mixture. The brown precipitate was filtered, washed with diethyl ether (2 × 25 mL) and dried under reduced vacuum (0.070 g, 74% yield.).

^{1}H NMR (500 MHz, d$_6$-acetone): δ 9.43 (t, J = 4.40 Hz, 1H, H_{phen}), 9.40 (d, J = 2.75 Hz, 1H, H_5), 8.92 (t, J = 7.48 Hz, 1H, H_{phen}), 8.86 (dd, J = 22.24, 7.35 Hz, 1H, H_{phen}), 8.77 (d, J = 8.26 Hz, 1H, H_{phen}), 8.55 (d, J = 7.68 Hz, 1H, H_4), 8.52 (t, J = 4.70 Hz, 1H, H_{phen}), 8.48–8.39 (m, 3H, H_{phen}), 8.32 (dd, J = 19.88, 7.47 Hz, 1H, H_{phen}), 8.22 (d, J = 8.86 Hz, 1H, H_{phen}), 8.19 (t, J = 7.54 Hz, 1H, H_3), 8.16–8.04 (m, 4H, $H_{1/phen}$), 7.98 (dd, J = 11.70, 8.89 Hz, 1H, H_{phen}), 7.76 (dd, J = 8.19, 5.28 Hz, 1H, H_{phen}), 7.53–7.45 (m, 2H, $H_{2/phen}$), 6.52 (dd, J = 8.36, 1.68 Hz, 2H, H_b), 6.40–6.35 (m, 2H, H_a), 3.35 (s, 1H, CH_2). LSI-MS: m/z 1734 $\{Ru_2(phen)_4\mathbf{L^1}(PF_6)_3\}^+$, 1589 $\{Ru_2(phen)_4\mathbf{L^1}(PF_6)_2\}^+$, 1443 $\{Ru_2(phen)_4\mathbf{L^1}(PF_6)\}^+$. ESI: m/z 1733 $\{Ru_2(phen)_4\mathbf{L^1}(PF_6)_3\}^+$. CHN: Calc. for $C_{73}H_{52}N_{12}Ru_2P_4F_{24}\cdot0.75(CH_3)_2CO$: C, 47.00; H, 2.96; N, 8.74%. Found: C, 46.95; H, 3.05; N, 8.49%. Selected IR data (cm^{-1}): 2846(br), 1500(w), 1427(m), 1411(w), 1208(w), 1147(w), 1036(w), 839(s), 769(m), 719(m), 660(w).

(Λ,Λ)-[Ru$_2$(phen)$_4$L^1](PF$_6$)$_4$

(Λ,Λ)-$[Ru_2(phen)_4\mathbf{L^1}](PF_6)_4$ was prepared by dissolving Λ-$[Ru(phen)_2(py)_2]$(arsenyl tartrate)$_2$ (0.21 g, 0.339 mmol) and **L^1** (0.040 g, 0.106 mmol)) in ethylene glycol (4 mL). The solution was heated at 120 °C for 6 h in darkness. After cooling to room temperature the solution was diluted with MeOH (6 mL). A saturated methanolic solution of NH_4PF_6 was added drop wise until no more precipitate formed. The precipitate was filtered, dried under reduced vacuum and recrystallised using an acetone–diethyl ether mixture. The brown precipitate was washed with diethyl ether (2 × 25 mL) and finally dried under reduced vacuum (0.174 g, 87% yield).

^{1}H NMR (400 MHz, d$_6$-acetone, Fig. 8): δ 9.43 (t, J = 4.02 Hz, 1H, H_{phen}), 9.41 (d, J = 2.36 Hz, 1H, H_5), 8.95–8.82 (m, 2H, H_{phen}), 8.77 (d, J = 8.23 Hz, 1H, H_{phen}), 8.56 (d, J = 7.85 Hz, 1H, H_4), 8.52 (t, J = 4.20 Hz, 1H, H_{phen}), 8.48–8.39 (m, 3H, H_{phen}), 8.32 (dd, J = 16.19, 8.07 Hz, 1H, H_{phen}), 8.25–8.03 (m, 6H, $H_{1/3/phen}$), 8.02–7.95 (m, 1H, H_{phen}), 7.76 (dd, J = 8.20, 5.26 Hz, 1H, H_{phen}), 7.54–7.45 (m, 2H, $H_{2/phen}$), 6.53 (d, J = 7.13 Hz, 2H, H_b), 6.38 (dd, J = 8.10, 5.64 Hz, 2H, H_a), 3.35 (s, 1H, CH_2). LSI-MS: m/z 1734 $\{Ru_2(phen)_4\mathbf{L^1}(PF_6)_3\}^+$, 1589 $\{Ru_2(phen)_4\mathbf{L^1}(PF_6)_2\}^+$, 1443 $\{Ru_2(phen)_4\mathbf{L^1}(PF_6)\}^+$. ESI: m/z 1733$\{Ru_2(phen)_4\mathbf{L^1}(PF_6)_3\}^+$, 795 $\{Ru_2(phen)_4\mathbf{L^1}(PF_6)_2\}^{2+}$. CHN: Calc. for $C_{73}H_{50}N_{12}Ru_2P_4F_{24}\cdot0.5(CH_3)_2CO$: C, 46.89; H, 2.91; N, 8.81%. Found: C, 47.11; H, 3.09; N, 8.61%. Selected IR data (cm^{-1}): 2347(w, br), 1601(w) 1501(w), 1427(m) 1250(m), 1206(w), 1016(w), 832(s), 768(m), 720(m), 658(w).

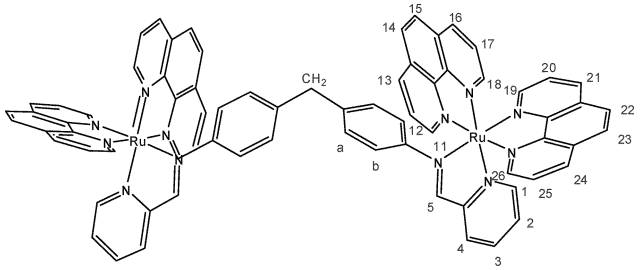

Fig. 8 $[Ru_2(phen)_4\mathbf{L^1}](PF_6)_4$, protons numbered.

(Δ,Δ)-[Ru$_2$(phen)$_4$L^1](PF$_6$)$_4$

(Δ,Δ)-$[Ru_2(phen)_4\mathbf{L^1}](PF_6)_4$ was prepared from Δ-$[Ru(phen)_2(py)_2]^{2+}$(arsenyl tartrate)$_2$ according to the procedure described

before for (Λ, Λ)-$[Ru_2(phen)_4\mathbf{L^1}]^{4+}(PF_6)_4$. The resulting precipitate was filtered, dried under reduced vacuum and recrystallised using an acetone–diethyl ether mixture. The brown precipitate was filtered, washed with diethyl ether (2 × 25 mL) and dried under reduced vacuum (0.176 g, 88% yield).

^{1}H NMR (400 MHz, d_6-acetone): δ 9.45–9.41 (m, 2H, $H_{5/phen}$), 8.89 (m, 2H, H_{phen}), 8.78 (dd, J = 8.25, 1.24 Hz, 1H, H_{phen}), 8.57 (d, J = 7.41 Hz, 1H, H_4), 8.55–8.51 (m, 1H, H_{phen}), 8.49–8.40 (m, 3H, H_{phen}), 8.36 (dd, J = 8.27, 1.20 Hz, 1H, H_{phen}), 8.26–8.04 (m, 6H, $H_{1/3/phen}$), 7.99 (d, J = 8.91 Hz, 1H, H_{phen}), 7.76 (dd, J = 8.21, 5.28 Hz, 1H, H_{phen}), 7.54–7.45 (m, 2H, $H_{2/phen}$), 6.56–6.51 (dd, 2H, H_b), 6.42–6.36 (m, 2H, H_a), 3.35 (s, 1H, CH_2). LSI-MS: m/z 1734 $\{Ru_2(phen)_4\mathbf{L^1}(PF_6)_3\}^+$, 1589 $\{Ru_2(phen)_4\mathbf{L^1}(PF_6)_2\}^+$, 1443 $\{Ru_2(phen)_4\mathbf{L^1}(PF_6)\}^+$. ESI: m/z 1733 $\{Ru_2(phen)_4\mathbf{L^1}(PF_6)_3\}^+$, 795 $\{Ru_2(phen)_4\mathbf{L^1}(PF_6)_2\}^{2+}$. CHN: Calc. for $C_{73}H_{50}N_{12}Ru_2P_4F_{24}\cdot 0.5(CH_3)_2CO$: C, 46.89; H, 2.91; N, 8.81%. Found: C, 46.61; H, 3.10; N, 8.53%. Selected IR data (cm^{-1}): 2348(w, br), 1602(w), 1504(w), 1427(m), 1205(w), 1016(w), 831(s), 763(m), 723(m), 660(w).

Cytotoxicity test

HBL100 and SKOV-3 cells were cultured according to the standard procedure, and maintained in a RPMI-1640 medium (Gibco) supplemented with 10% FBS (Invitrogen), 2 mM l-glutamin (Sigma), 1 mM sodium pyruvate (Sigma), 10 mM Hepes buffer (Sigma) and antibiotics (Antibiotics Antimycotic 100×, diluted to 1× with buffer, Sigma). Cells from confluent monolayers were removed from flasks by 1% trypsin (trypsin-EDTA 10× was diluted to 1× using PBS, Sigma). Cell viability was determined by the trypan blue dye exclusion test.

For the cytotoxicity evaluation for HBL100 and SKOV-3 10 000 cells a well were seeded, both cell lines in 100 µl of complete medium in 96-multiwell flatbottom microtiter plates (Costar). The plates were incubated at 37 °C, 5% CO_2 for 24 h prior to drug testing to allow cell adhesion. The stock solutions of the $[\{Ru(bpy)_2\}_2L](PF_6)_4$ compounds (0.4 mg mL^{-1}, 2% dmso in medium) and $[\{Ru(phen)_2\}_2L](PF_6)_4$ compounds (0.3 mg mL^{-1}, 2% dmso in medium) were freshly prepared and directly used for dilutions. The dilutions were prepared in complete medium. The range of final concentrations (in well) for $[\{Ru(bpy)_2\}_2L](PF_6)_4$ compounds was 0.2, 0.1, 0.04, 0.02, 0.004 mg mL^{-1}. For the $[\{Ru(phen)_2\}_2L](PF_6)_4$ compounds the range of final concentrations was 0.3, 0.15, 0.06, 0.03, 0.006 mg mL^{-1}. As a control cisplatin was used and was dissolved in the complete medium and further diluted. Each concentration was tested in quadruplicate using 100 µl/well added to the 100 µl of cells in complete medium. In the control group only 100 µl of complete medium was added with 2% of DMSO. The plates were incubated for 72 h and the evaluation of cell proliferation was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) colorimetric assay. 20 µl MTT solution (5 mg mL^{-1} in PBS, Sigma) was added to each well and incubated for 2 h. Formazan crystals were solubilized in 200 µl dmso. Optical density was measured using a Bio-Tek FL600 plate reader (Bio-Tek Instruments Inc., Vermont, USA) at 590 nm. IC_{50} values were obtained by GraphPad Prism software, version 3.05, 2000.

Acknowledgements

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