# Folates are potential ligands for ruthenium compounds in vivo

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Supporting Information

## Contents

# Experimental

## *Materials*

[$RuCl_3 \cdot 3H_2O$] was obtained from Oxkem Limited (Reading, UK). "Ultrapure water" (with a resistivity of 18.2 MΩ cm or greater) was obtained from a Barnstead Nanopure ultrapure water system (Thermo Scientific, Basingstoke, UK) and passed through a 0.22 μm membrane filter prior to use. HPLC grade acetonitrile was obtained from Fischer Scientific (Loughborough, UK) and used as received. All other reagents and NMR spectroscopy solvents were obtained from Sigma-Aldrich (Dorset, UK) and used as received.

## *Instrumentation*

### NMR Spectroscopy

$^1$H NMR spectra and homo and hetero nuclear 2D-NMR spectra of the [*cis*-Ru(2,2'-bipy)$_2$(folic acid)(PF$_6$)$_2$] were carried out on an Avance 700 FT-NMR spectrometer, all other spectra collected on a DRX-500 FT-NMR spectrometer ($^{13}$C NMR spectra with broadband decoupling).

### Mass Spectrometry (MS)

Samples studied by electrospray ionisation MS (ESI-MS) were typically analysed in water or water:acetonitrile solutions (1:1 by volume). Samples were made up to a concentration of 1-50 μM, injected into the capillary of the micromass Quattro LC at a rate of 10 μL per minute and collected with a capillary voltage of 2.8 kV, cone voltage of 30 V and a collection voltage of 3 V. Desolvation and capillary temperatures were at 40 °C. Ruthenium containing species are particularly distinctive in the mass spectra due to their isotope pattern.[1] Alterations to this isotope pattern allow for accurate analysis of such peaks. Alterations may be due to an increased charge or association of atoms with their own isotope patterns (e.g. the 3:1 ratio of $^{35}$Cl to $^{37}$Cl). Where values have been quoted, this will correspond to the peak of highest intensity, therefore containing $^{102}$Ru (as well as the most abundant combination of isotopes of any other elements present). Hence, in attempted syntheses that resulted in a mixture of products, the mass spectra assignments are consistent with the mass-charge ratio as well as the isotope patterns observed. Without isolation of the separate species these assignments are only the best possible suggestions and have not been confirmed by further analytical techniques. All $^m/_z$ values have been given to the nearest 0.1 Daltons per unit charge.

## UV-Vis Spectroscopy

Samples were dissolved in ultrapure water and transferred to a quartz cuvette of path length 1.0 cm (Starna Scientific, Essex, UK). Spectra were acquired using a Varian Cary 100 Spectrophotometer (Varian Ltd., Oxford, UK) at 298 K, with the instrument being zeroed with ultrapure water. Spectra were acquired at 1.0 nm intervals using a spectral bandwidth of 2 nm.

## IR Spectroscopy

Solid was loaded directly onto a PerkinElmer Spectrum TWO FT-IR machine (PerkinElmer, Cambridge, UK) and analysed using the Spectrum software package.

# *Protocol*

## Synthesis of [*cis*-Ru(2,2'-Bipy)$_2$Cl$_2$·2H$_2$O]

Adapted from literature prep.[2]
[$RuCl_3 \cdot 3H_2O$] (3.86 g, 14.8 mmol), 2,2'-bipyridine (4.68 g, 30.0 mmol) and LiCl (4.23 g, 101 mmol) were dissolved in DMF (25 mL) and heated under reflux overnight. The reaction mixture was cooled to room temperature before acetone (100 mL) was added and the mixture stored at 4 °C for 48 hours. The reaction mixture was filtered and the precipitate washed with ice cold water (5 x 7 mL), ether (10 mL), followed by a solution of LiCl (8.0 g in 40 mL of water) (5 x 8 mL) and ether (10 mL) before being dried *in vacuo*.

Yield: (5.25 g, 10.1 mmol, 68 %)

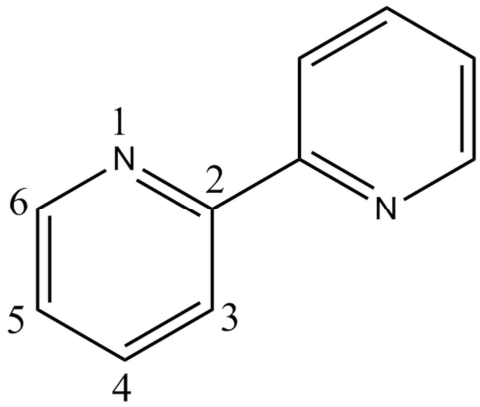
$^1$H NMR: (300 K, $(CD_3)_2SO$) δ = 9.96 (dd, 4J(H,H) = 0.8 Hz, 3J(H,H) = 5.6 Hz; 2H; bipy-6'), 8.64 (d 3J(H,H) = 8.0 Hz; 2H; bipy-3'), 8.48 (d 3J(H,H) = 7.9 Hz; 2H; bipy-3), 8.06 (dt, 4J(H,H) = 1.5 Hz, 3J(H,H) = 7.8 Hz; 2H; bipy-4'), 7.76 (dt; 4J(H,H) = 1.4 Hz, 3J(H,H) = 5.8 Hz, 2H; bipy-5'), 7.67 (dt, 4J(H,H) =1.4 Hz, 3J(H,H) = 7.5 Hz; 2H; bipy-4), 7.498 (dd, 4J(H,H) = 0.6 Hz, 3J(H,H) = 6.5 Hz; 2H; bipy-6), 7.10 (dt, 4J(H,H) = 1.3 Hz, 3J(H,H) = 5.9 Hz, 2H; bipy-5).

$^{13}$C NMR: (300 K, $(CD_3)_2SO$) δ = 160.3 (bipy-2), 158.3 (bipy-2'), 153.3 (bipy-6'), 152.1 (bipy-6), 134.7 (bipy-4'), 133.5 (bipy-4), 125.5 (bipy-5'), 125.4 (bipy-5), 123.0 (bipy-3), 122.6 (bipy-3').

$m/z$ = 484 (singly charged, [*cis*-Ru(2,2'-Bipy)$_2$Cl$_2$]$^+$), 490 (singly charged, [*cis*-Ru(2,2'-Bipy)$_2$(NCCH$_3$)Cl]$^+$)

$\lambda_{max}$ = 487 nm

Figure S-1: Numbering system of pyridine rings as applied to all 2,2'-bipyridine ligands herein.




## Synthesis of 10-Formyl Folic Acid and 10-Formyl Pteroic Acid

Adapted from literature prep.[3]
Formic acid (40 mL) was dried with $MgSO_4$ (~0.5 g) until the $MgSO_4$ was free flowing. Folic acid (400 mg, 0.907 mmol) was added and the mixture heated under reflux for one hour. The reaction was quenched on ice before ether (60 mL) was added forming an orange precipitate which was collected, washed with methanol (3 x 10 mL) followed by ether (2 x 10 mL) and dried *in vacuo*.

$^1$H NMR: (298 K, $(CD_3)_2SO$) δ =8.81(s; PA formyl-**H**), 8.77 (s; FA formyl-**H**), 8.64 (s; FA-7 + PA-7), 8.60 (m; FA-18), 7.90 (d, 3J = 8.5 Hz; PA-13/15), 7.89 (d, 3J = 8.5 Hz; FA-13/15), 7.56(d, 3J = 8.5 Hz; PA-12/16), 7.54 (d, 3J = 8.5 Hz; FA-12/16), 5.18 (s; FA-9+PA-9), 4.37 (m; FA-19), 2.34 (t, 3J = 7.5 Hz; FA-22), 2.05 (m; FA-21ii), 1.93 (m; FA-21i)
Integrals suggest ~3:2 folic acid (FA): pteroic acid (PA)

$m/z$ = 341.1 ([10-formyl pteroic acid + H]$^+$), 470.2 ([10-formyl folic acid + H]$^+$)

## Synthesis of [*cis*-Ru(2,2'-bipy)$_2$(folic acid)(PF$_6$)$_2$]

[*cis*-Ru(2,2'-Bipy)$_2$Cl$_2$·2H$_2$O] (108 mg, 0.208 mmol) and folic acid (89 mg, 0.202 mmol) were suspended in water (30 mL) and heated to 65 °C overnight turning the suspension into a red/brown solution. This mixture was reduced in volume to *circa* 7 mL before $NH_4PF_6$ (98 mg, 0.601 mmol) was added resulting in a dark precipitate of two pairs of diastereomers (x = ΛR, ΔS; y = ΛS, ΔR; x:y ~ 1:2 by NMR spectroscopy integrals) which was collected, washed with ether (3 x 10 mL) and dried *in vacuo*.

Yield: (137 mg, 0.120 mmol, 59 %)

Elemental analysis:
Theoretical:

C=40.91 % H=3.08 % N=13.46 %

Results:
C=41.06 % H=3.20 % N=13.36 %  (2.0806 mg)
C=40.92 % H=3.15 % N=13.25 %  (1.6554 mg)

$^{1}$H NMR: (298 K, $(CD_3)_2SO$) δ = 8.83 (d, 3J = 8.4 Hz; bipy-3), 8.73 (m; bipy-3), 8.71 (m; bipy-3), 8.66 (s; FA-7x), 8.64 (s; FA-7y), 8.44 (m; bipy-6), 8.32 (d, 3J = 4.2 Hz; bipy-6), 8.30 (m; bipy-3), 8.27 (m; bipy-4), 8.24 (t, 3J = 8.4 Hz; bipy-4), 8.15 (t, 3J = 7.0 Hz, FA-13/15), 8.02 (m; bipy-4), 7.80 (t, 3J = 6.3 Hz; bipy-5), 7.78 (d, 3J = 7.7 Hz; bipy-6y), 7.76 (d, 3J = 7.0 Hz; bipy-6x), 7.65 (t, 3J = 7.0 Hz; bipy-5), 7.56 (t; 3J = 4.6 Hz; bipy-4y), 7.52 (m; bipy-4x), 7.52 (d, 3J = 9.1 Hz; FA-13/15), 7.39 (m; bipy-5), 7.38 (m; bipy-6), 7.10 (t, J = 7.0 Hz; bipy-5y), 7.08 (m; bipy-5x), 6.23 (t, 3J = 4.6 Hz; FA-10x), 6.08 (d,d 3J = 11.9 Hz, 3J = 7.0 Hz; FA-10y), 5.90 (m; FA-12/16), 4.34 (m; FA-19), 3.90 (m; FA-9ix), 3.87 (m; FA-9iy), 3.23 (m; FA-9iix), 3.20 (m; FA-9iiy), 2.32 (d,d 3J = 12.6 Hz, 3J = 7.7 Hz; FA-22), 2.04 (m; FA-21ii), 1.90 (m; FA-21i)

$^{13}$C NMR: (300 K, $(CD_3)_2SO$) δ = 178.2 (FA-2/4/8a), 174.0 (FA-23), 173.9 (FA-20x+y), 167.4 (FA-2/4/8a), 166.4 (FA-17x/y), 166.3 (FA-17x/y), 158.5 (bipy-2x/y), 158.5 (bipy-2x/y), 157.5 (bipy-2), 157.3 (bipy-2), 157.0 (bipy-2), 153.1 (bipy-6 + FA-6), 152.8 (bipy-6x+y), 151.9 (FA-7x), 151.8 (FA-7y), 151.1 (bipy-6), 150.5 (FA-2/4/8a), 150.4 (bipy-6), 149.4 (FA-11), 137.8 (bipy-4), 137.7 (bipy-4 + bipy-4), 135.8 (bipy-4), 130.9 (FA-13/15), 129.2 (FA-4a), 128.8 (FA-13/15), 127.8 (bipy-5), 127.5 (bipy-5), 127.3 (bipy-5), 126.8 (bipy-5), 124.1 (bipy-3), 123.8 (bipy-3), 123.8 (bipy-3), 123.6 (bipy-3x+y), 122.1 (FA-14x/y), 118.6 (FA-14x/y), 111.1 (FA-12/16), 111.0 (FA-12/16), 51.9 (FA-19), 45.5 (FA-9x/y), 45.5 (FA-9x/y), 30.5 (FA-22), 26.1 (FA-21).

$m/z$ = 363.1 ([*cis*-Ru(2,2'-Bipy)$_2$(pteroic acid)]$^{2+}$), 427.7 ([*cis*-Ru(2,2'-Bipy)$_2$(folic acid)]$^{2+}$), 706.7 ([*cis*-Ru(2,2'-Bipy)$_2$)$_2$(folic acid-H$^+$)][$PF_6$]$^{2+}$), 725.1 ([*cis*-Ru(2,2'-Bipy)$_2$(pteroic acid-H$^+$]$^+$), 854.0 ([*cis*-Ru(2,2'-Bipy)$_2$(folic acid-H$^+$]$^+$), 1000.1 ([*cis*-Ru(2,2'-Bipy)$_2$(folic acid)][$PF_6$]$^+$).

$\lambda_{max}$ = 470 nm

IR = 762 $cm^{-1}$ (sharp, strong), 834 $cm^{-1}$ (sharp, strong), 1605 $cm^{-1}$ (sharp, medium), 1655 $cm^{-1}$ (sharp, medium)

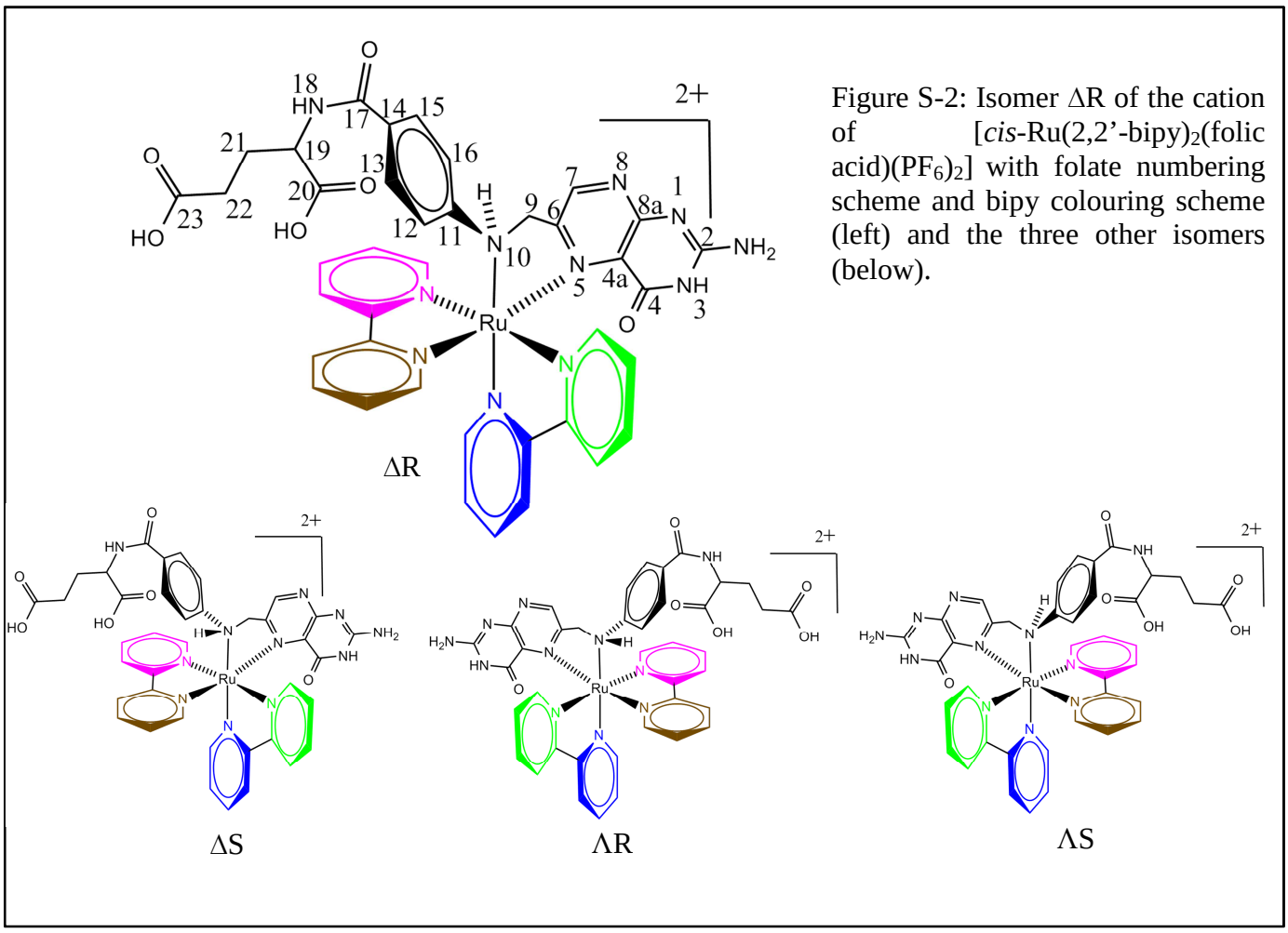



Figure S-2: Isomer ΔR of the cation of [*cis*-Ru(2,2'-bipy)$_2$(folic acid)$(PF_6)_2$] with folate numbering scheme and bipy colouring scheme (left) and the three other isomers (below).

Figure S-3: UV-Vis spectra of the reactant and product ruthenium compounds in water.

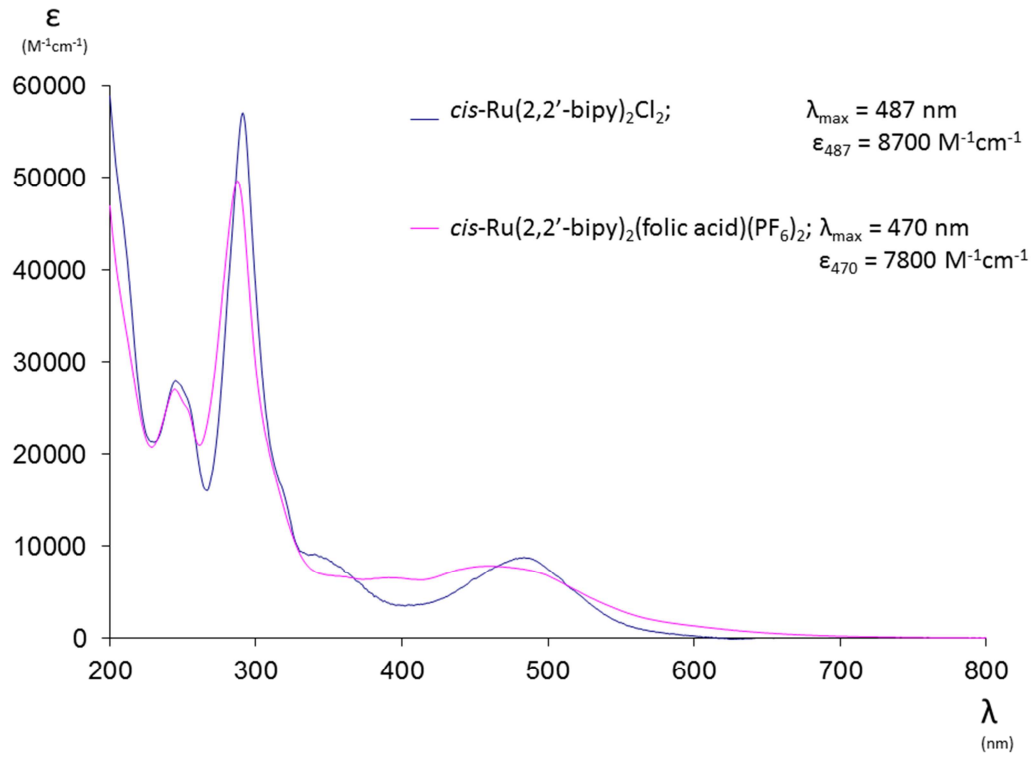
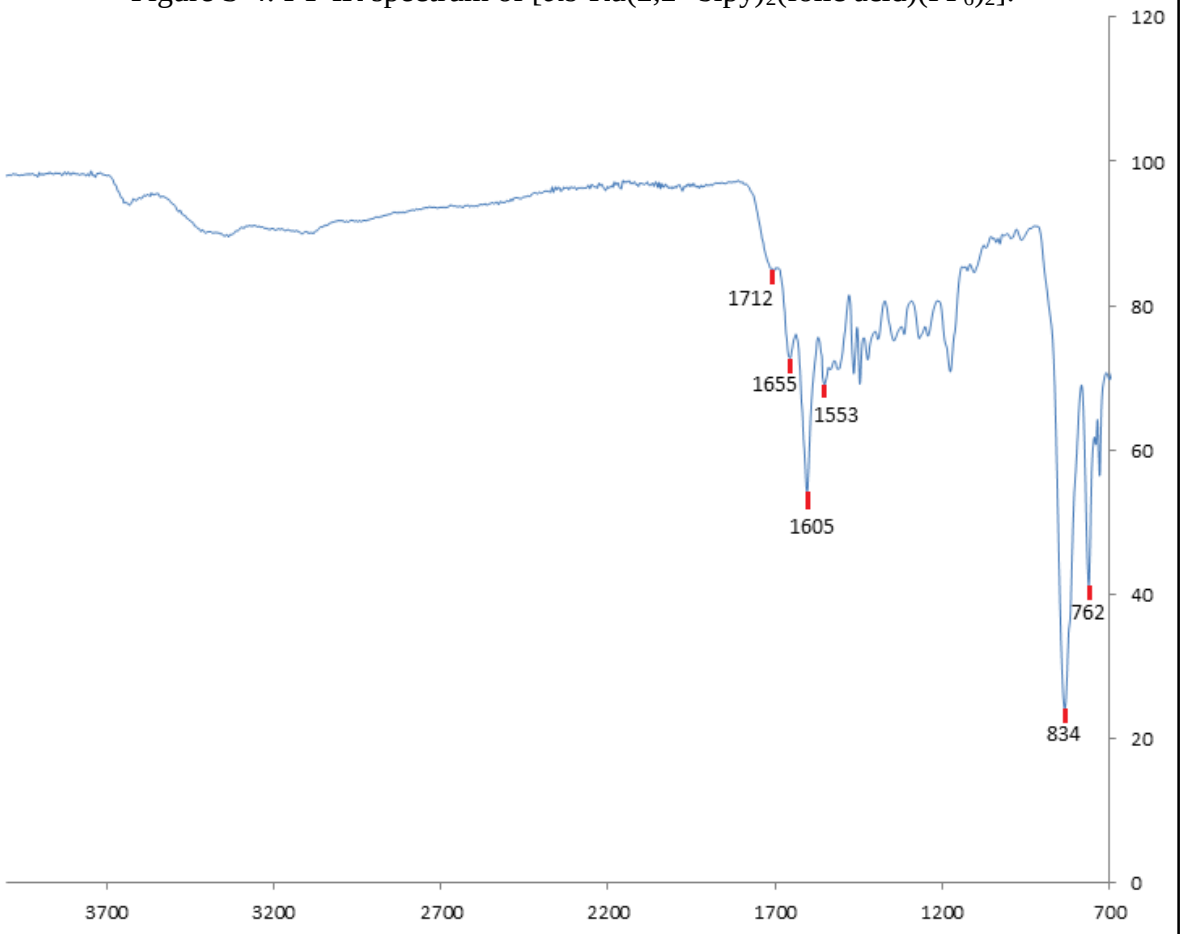



Figure S-4: FT-IR spectrum of [*cis*-Ru(2,2'-bipy)$_2$(folic acid)(PF$_6$)$_2$].




Above 1000 cm$^{-1}$, the IR spectrum of [*cis*-Ru(2,2'-bipy)$_2$(folic acid)(PF$_6$)$_2$] shows great similarity to that of free folic acid,[4] however, the stretching frequencies in the carbonyl region are noticeably changed. The peaks at 834 cm$^{-1}$ and 762 cm$^{-1}$ are typical of metal ligand coordination bonds.

[*cis*-Ru(2,2'-bipy)$_2$(folic acid)(PF$_6$)$_2$] 2D-NMR spectral assignments and correlations are given in the spreadsheets of appendix 2. The spectra are reproduced in appendix 1. Building a 3D model of the cation was a crucial aid in testing the feasibility and validity of these assignments.

Equivalent procedures were carried out in citrate buffers (pH 2.5 and 6.0) and CAPS buffer (pH 9.9). All buffers were 10 mM and the folate was only observed to bind at pH 6.0 and 9.9 as determined by MS. NMR spectroscopy suggested these products were isolated in a lower purity than the unbuffered reaction. The equivalent procedure was also performed un-buffered, with 10-formyl folic acid resulting in only starting materials being detected by MS.

## Synthesis of [*cis*-Ru(2,2'-bipy)$_2$(folic acid)(PF$_6$)$_2$] following the previously published procedure[5]

[*cis*-Ru(2,2'-Bipy)$_2$Cl$_2$·2H$_2$O] (50 mg, 0.096 mmol) and folic acid (44 mg, 0.100 mmol) were suspended in ethanol/water (1:5, 15 mL) and heated under reflux overnight turning the suspension into a red/brown solution. The mixture was cooled to room temperature before $NH_4PF_6$ (33 mg, 0.202 mmol) was added and being stored at 4 °C overnight. The resulting precipitate was collected, washed with ice cold ethanol/water (3 mL) and dried *in vacuo*.

Yield: (10 mg, 0.009 mmol, 9 %)

$^1$H NMR: Signals were as for the product of the procedure outlined above although the integrals suggested the ΛS, ΔR isomers were further favoured.
(x = ΛR, ΔS; y = ΛS, ΔR; x:y ~ 1:4 by NMR spectroscopy integrals)

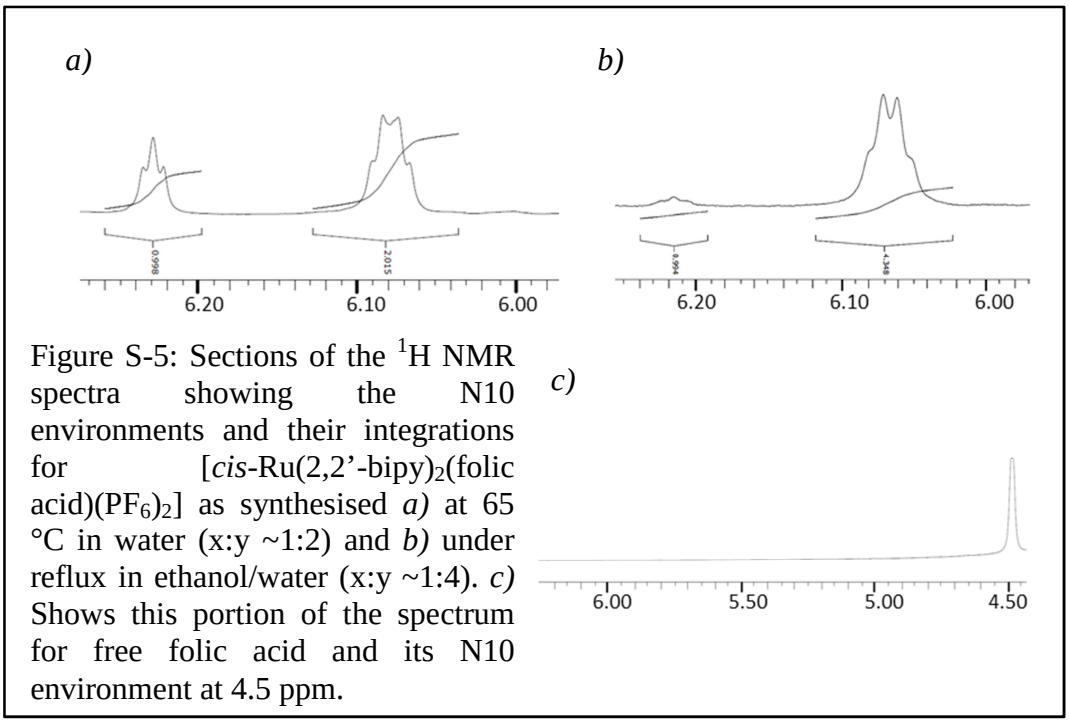



Figure S-5: Sections of the $^1$H NMR spectra showing the N10 environments and their integrations for [*cis*-Ru(2,2'-bipy)$_2$(folic acid)(PF$_6$)$_2$] as synthesised *a)* at 65 °C in water (x:y ~1:2) and *b)* under reflux in ethanol/water (x:y ~1:4). *c)* Shows this portion of the spectrum for free folic acid and its N10 environment at 4.5 ppm.

## *Buffer Recipes*

### Phosphate buffered saline (PBS)

### pH 7.35

10 mM phosphate solution was prepared at pH 7.35 using mono and di basic sodium phosphate salts with 137 mM NaCl and 2.7 mM KCl added.

### Citrate buffer

### pH 2.5

Citric acid (1.901 g, 9.89 mmol) and trisodium citrate dihydrate (0.183 g, 0.622 mmol) were dissolved in ultrapure water (990 mL). The pH was adjusted to the desired level with strong acid/base (1 M HCl/NaOH) as required before the total volume was made up to 1000 mL.

### pH6.0

Citric acid (0.265 g, 1.38 mmol) and trisodium citrate dihydrate (2.570 g, 8.74 mmol) were dissolved in ultrapure water (990 mL). The pH was adjusted to the desired level with strong acid/base as required before the total volume was made up to 1000 mL.

### CAPS buffer

### pH9.9

N-cyclohexyl-3-aminopropanesulfonic acid (2.235 g, 10.10 mmol) was dissolved in ultrapure water (990 mL) and the pH adjusted to the desired level with 1 M NaOH before the total volume was made up to 1000 mL.

## *Normothermic Reactions of [cis-Ru(2,2'-Bipy)$_2$Cl$_2$·2H$_2$O]*

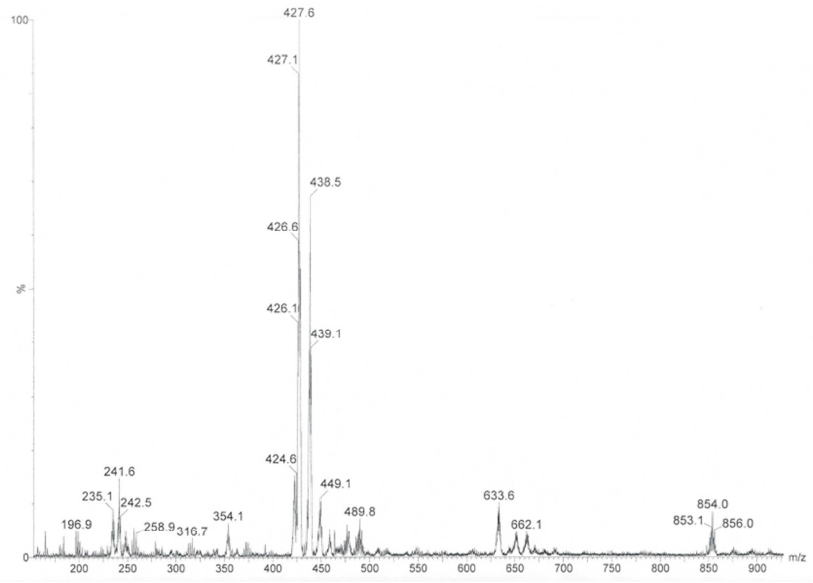
### With Folic Acid in PBS

PBS (10 mL) was added to Folic acid (22 mg, 0.050 mmol) and [*cis*-Ru(2,2'-Bipy)$_2$Cl$_2$·2H$_2$O] (26 mg, 0.050 mmol) before heating to 37 °C with stirring overnight.

For a sample suitable for NMR spectroscopy, the solvent was removed under vacuum at room temperature, ruthenium complexes dissolved in methanol and the mixture filtered. The solvent was then removed from the filtrate under vacuum at room temperature and the resultant residue dissolved in $(CD_3)_2SO$. Diagnostic signals in a relatively clear part of the spectrum were identifiable as follows:
$^1$H NMR: (298 K, $(CD_3)_2SO$) δ = 6.30 (d,d 3J = 10.3 Hz; 3J = 5.0 FA-10x), 6.08 (m FA-10y), 3.79 (d 2J = 13.2 Hz FA-9ix), 3.59 (m FA-9iy), 3.16 (m FA-9iix), 3.06 (m FA-9iiy)
x:y, ~3:2 by FA-10 integrals
FA-10 integrals suggest ~90 % of mixture is FA coordinated complex relative to *cis*-Ru(2,2'-Bipy)$_2$Cl$_2$ starting material present (diagnostic signals at > 9.5 ppm).

$m/z$ = 854.0 ([*cis*-Ru(2,2'-bipy)$_2$(folic acid-H$^+$]$^+$), 633.6 ([(*cis*-Ru(2,2'-bipy)$_2$)$_2$(folic acid-2H$^+$]$^{2+}$), 427.6. ([*cis*-Ru(2,2'-bipy)$_2$(folic acid]$^{2+}$), 438.5 ([*cis*-Ru(2,2'-bipy)$_2$(folic acid-H$^+$+Na$^+$]$^{2+}$), 449.1 ([*cis*-Ru(2,2'-bipy)$_2$(folic acid-2H$^+$+2Na$^+$)]$^{2+}$) and other minor species corresponding to [*cis*-Ru(2,2'-bipy)$_2$L$^1$L$^2$]$^{n+}$ where L$^1$ and L$^2$ are various combinations of monodentate solvent and chloride ligands resulting in either singly or doubly charged species.

Figure S-6: ESI-MS of the reaction mixture of folic acid and [*cis*-Ru(2,2'-Bipy)$_2$Cl$_2$·2H$_2$O] in PBS after 1 day (diluted to approximately 10 μM in 50:50 acetonitrile:water).




## With Folic Acid

Folic acid (42 mg, 0.095 mmol) was added to ultrapure water (20 mL) and warmed into solution. This solution was cooled to room temperature before [*cis*-Ru(2,2'-Bipy)$_2$Cl$_2$·2H$_2$O] (50 mg, 0.096 mmol) was added and the mixture maintained at 37 °C with stirring. Aliquots were taken immediately, after 1 hour, 3 hours, 6 hours and then daily for 6 days.

$m/z$ = 854.2 ([*cis*-Ru(2,2'-bipy)$_2$(folic acid-H$^+$)]$^+$), 633.7 ([(*cis*-Ru(2,2'-bipy)$_2$)$_2$(folic acid-2H$^+$)]$^{2+}$) and signals corresponding to starting materials.

## With Dihydrofolate (DHF)

DHF (25 mg, 0.056 mmol) was added to ultrapure water (12.5 mL) and warmed into solution. Half of this solution was taken, cooled to room temperature and added to [*cis*-Ru(2,2'-Bipy)$_2$Cl$_2$·2H$_2$O] (15 mg, 0.029 mmol) before being stirred at 37 °C in the dark. Aliquots were taken after 1 and 2 days.

$m/z$ = 428.7 ([*cis*-Ru(2,2'-bipy)$_2$(DHF)]$^{2+}$), 427.7 ([*cis*-Ru(2,2'-bipy)$_2$(folic acid)]$^{2+}$) and signals corresponding to starting materials.

### NMR Assignment of Folic Acid

$^1$H NMR: (298 K, $(CD_3)_2SO$) δ = 8.63 (s; FA-7), 8.17 (d, 3J = 9.5 Hz; FA-18), 7.62 (d, 3J = 11.0 Hz; FA-13/15), 6.92 (broad s; FA-10), 6.63 (d, 3J = 11.0 Hz; FA-12/16), 4.48 (broad s; FA-9), 4.32 (m; FA-19), 2.30 (t, 3J = 9.0 Hz; FA-22), 2.04 (m; FA-21ii), 1.90 (m; FA-21i)

$^{13}$C NMR: (300 K, $(CD_3)_2SO$) δ = 174.6 (FA-23), 174.3 (FA-20), 167.2 (FA-17), 161.9 (FA-2/4), 156.6 (FA-2/4a/8a), 154.2 (FA-4/8a), 151.3 (FA-11), 149.2 (FA-7+ FA-6), 129.5 (FA-13/15), 128.2 (FA-4a/8a), 121.6 (FA-14), 111.8 (FA-12/16), 52.3 (FA-19), 46.2 (FA-9), 30.9 (FA-22), 26.4 (FA-21)

(Numbering system as for the folate in the coordinated species.)

## References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2. B.P. Sullivan, D.J. Salmon, T.J. Meyer, *Inorg. Chem.*, 1978, 17, 3334-3341.
3. Merck Eprova AG, R. Moser, R. Schibli, C.M. Müller, V. Groehn, U. Michel, C. Sparr, T.L. Mindt, World Intellectual Property Organisation, 2007, WO 2008/125618 A1
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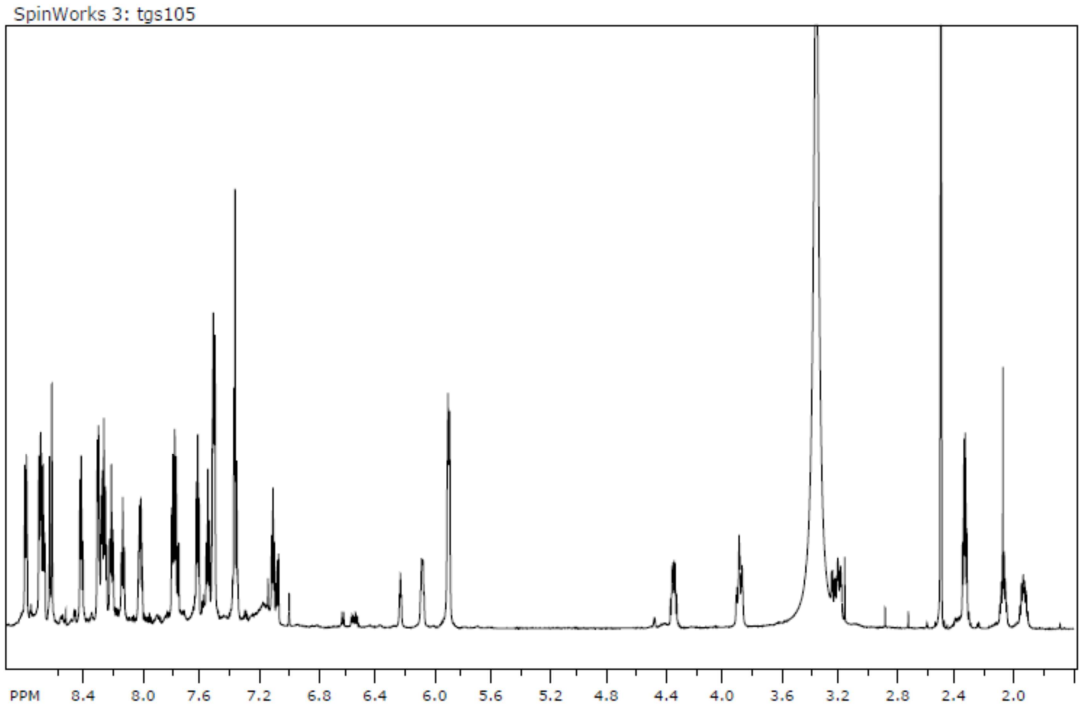
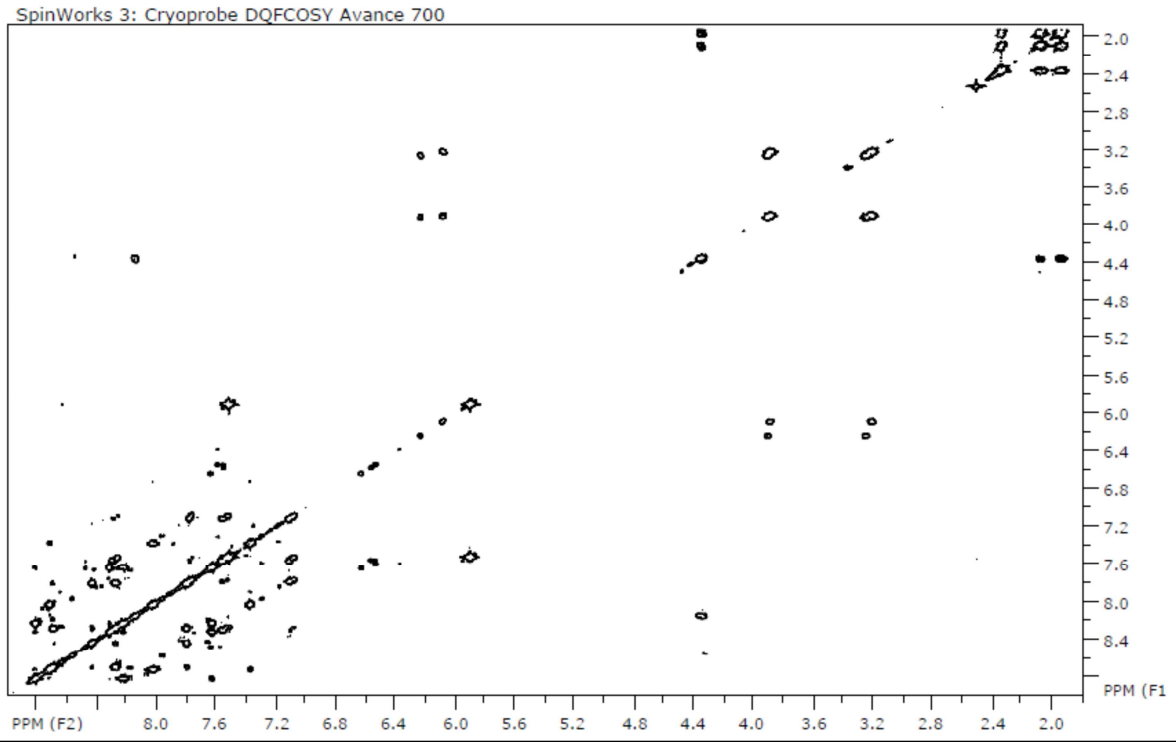
5. B. Schwederski, W. Kaim, *Inorganica Chimica Acta*, 1992, **195**, 123-126.

# Appendix 1: NMR Spectra of [cis-Ru(2,2'-bipy)$_2$(folic acid)($PF_6$)$_2$]

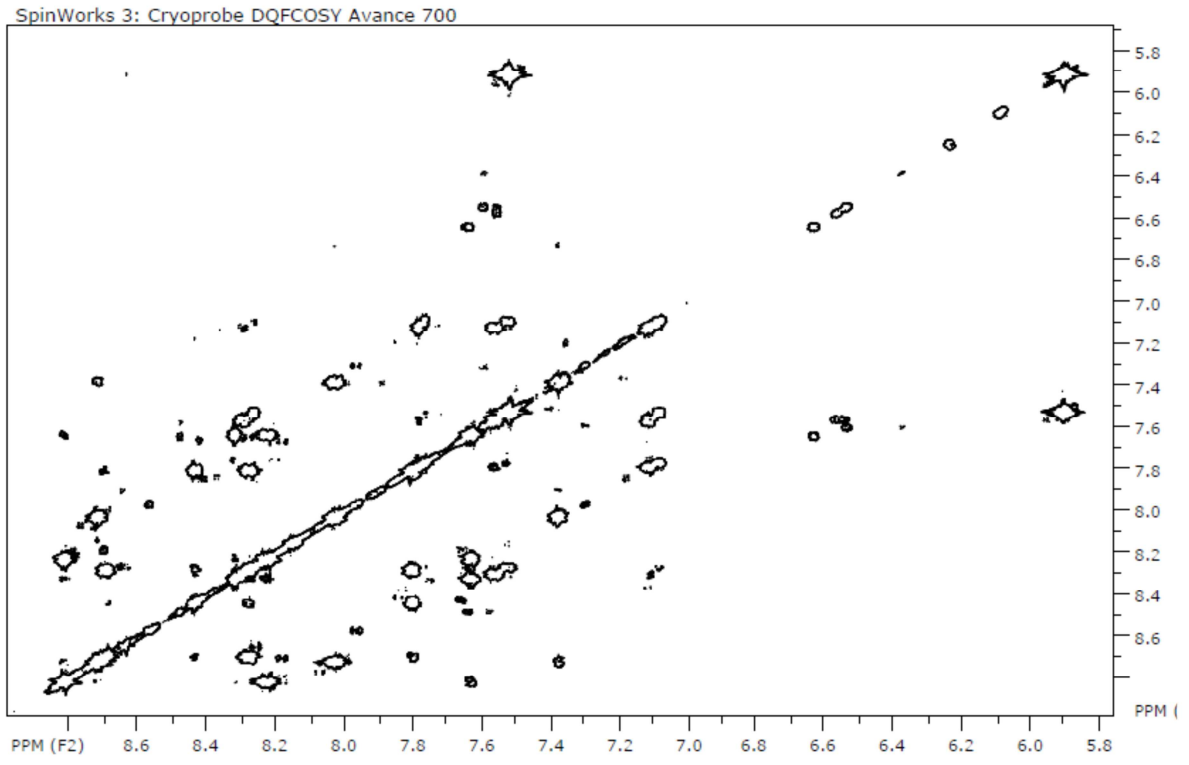
## $^1$H NMR Spectrum



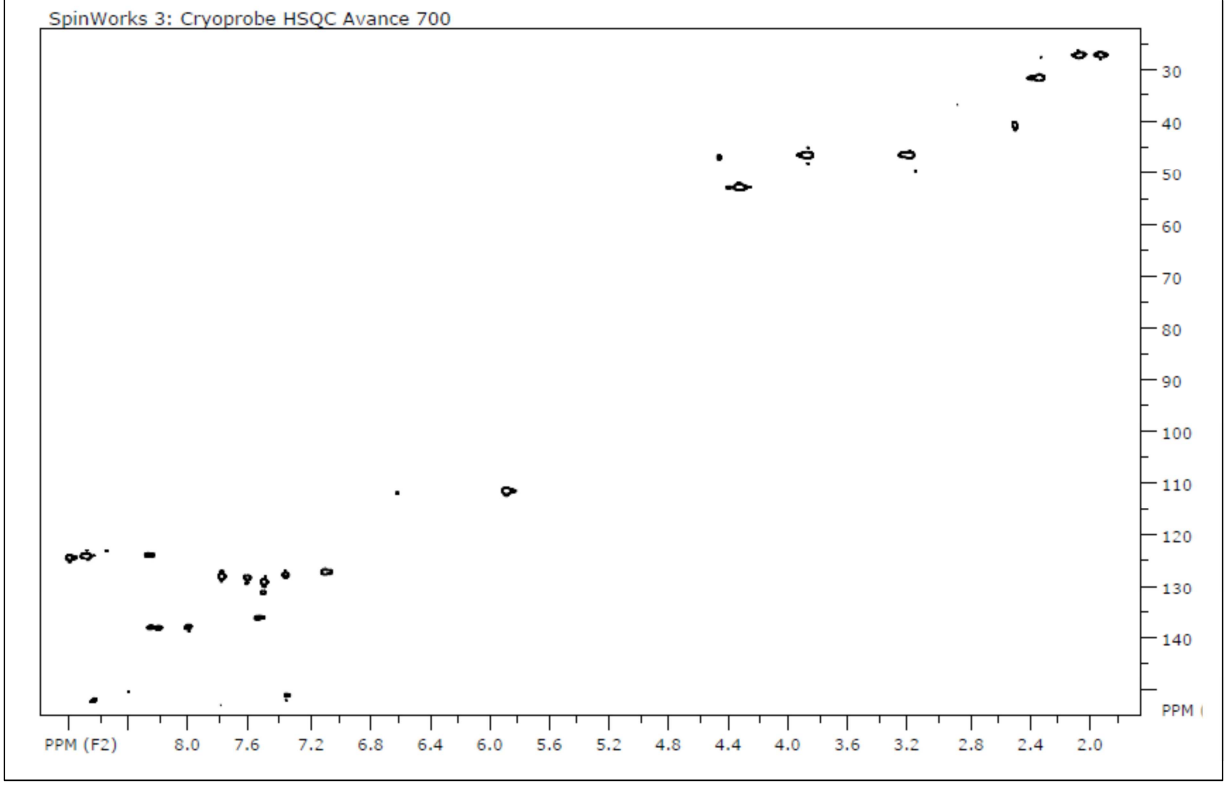

## $^1$H COSY NMR Spectrum



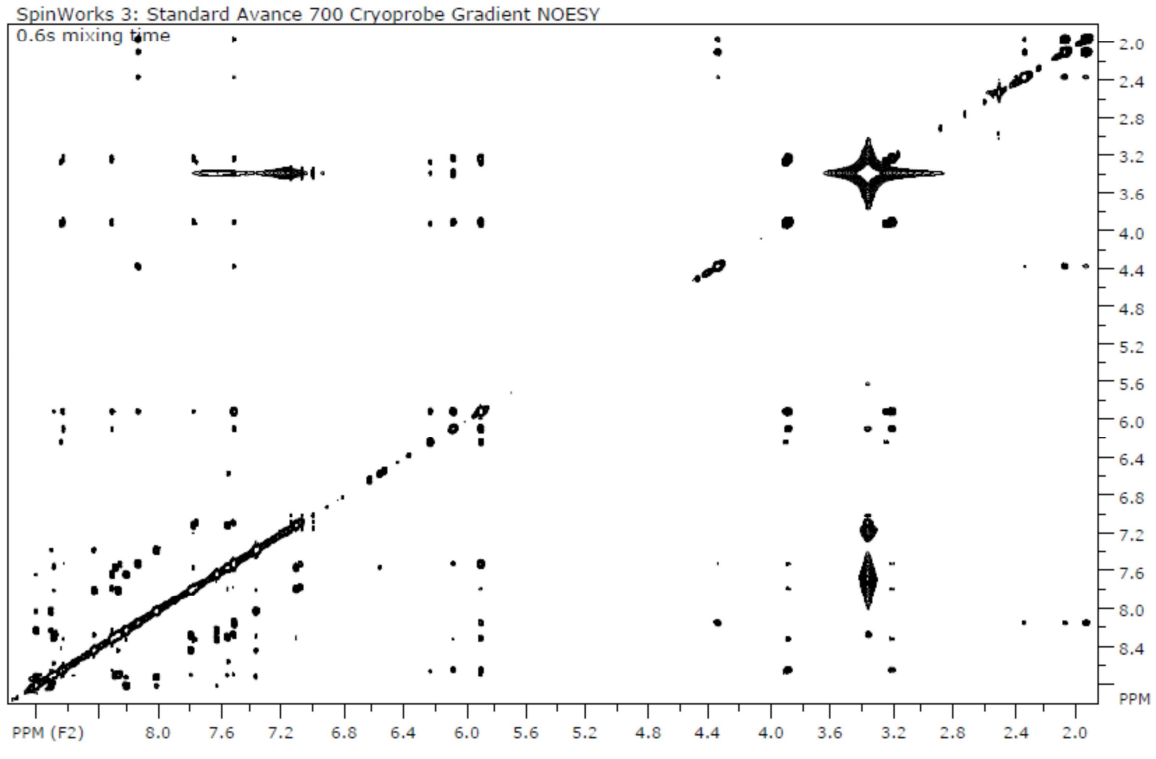
## $^{1}$H COSY NMR Spectrum: downfield region




## Heteronuclear NMR Spectrum: HSQC



## $^{1}$H NOESY NMR Spectrum



# Appendix 2: 2D NMR Analysis and Assignments of $[Ru(bipy)_2(folic\ acid)(PF_6)_2]$

| | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Key | Signal Strength | | W= | weak | v.= | very | | FA= | folic acid | | | Isomers | | x= | ΛR;ΔS | | | | | | | |
| | | | | | M= | medium | | | | BIPY= | 2,2'-bipyridine | | | (x:y , 1:2) | | y= | ΛS;ΔR | | | | | | | |
| | | | | | S= | strong | | | | ?= | uncertain assignment* | | | | | | | | | | | | | |
| | | | Colour coding of bipyridine ligands as outlined in figure S-2 | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | | | | |
| | | | *assignments have been labelled as uncertain when: | | | | | | | | | | | | | | | | | | | | | |
| | | | a) there is ambiguity due to signals of similar shifts. These are: | | | | | | | | Bipy-5 (7.39 ppm) and Bipy-6 (7.38 ppm) | | | | | | | | | | | | | |
| | | | | | | | | | | | Bipy-4x (7.52 ppm) and FA-13/15 | | | | | | | | | | | | | |
| | | | b) the signal is so weak it may be attributable to noise | | | | | | | | | | | | | | | | | | | | | |

## Table S-1: Bipyridine/Bipyridine COSY Crosspeaks

| | Shift | Assignment | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Shift | | | 8.83 | 8.24 | 7.65 | 8.32 | 8.73 | 8.02 | 7.39 | 7.38 | 8.71 | 8.27 | 7.80 | 8.44 | 8.30 | 7.56 | 7.10 | 7.78 | | | |
| Assignment | | | Bipy-3 | Bipy-4 | Bipy-5 | Bipy-6 | Bipy-3 | Bipy-4 | Bipy-5 | Bipy-6 | Bipy-3 | Bipy-4 | Bipy-5 | Bipy-6 | Bipy-3 | Bipy-4x+y | Bipy-5x+y | Bipy-6x+y | | | |
| | 8.83 | Bipy-3 | | S | W | | | | | | | | | | | | | | | | |
| | 8.24 | Bipy-4 | S | | S | | | | | | | | | | | | | | | | |
| | 7.65 | Bipy-5 | W | S | | S | | | | | | | | | | | | | | | |
| | 8.32 | Bipy-6 | | | S | | | | | | | | | | | | | | | | |
| | 8.73 | Bipy-3 | | | | | | S | M | | | | | | | | | | | | |
| | 8.02 | Bipy-4 | | | | | S | | S | | | | | | | | | | | | |
| | 7.39 | Bipy-5 | | | | | M | S | | S | | | | | | | | | | | |
| | 7.38 | Bipy-6 | | | | | | | S | | | | | | | | | | | | |
| | 8.71 | Bipy-3 | | | | | | | | | | S | | | | | | | | | |
| | 8.27 | Bipy-4 | | | | | | | | | S | | S | W | | | | | | | |
| | 7.80 | Bipy-5 | | | | | | | | | | S | | S | | | | | | | |
| | 8.44 | Bipy-6 | | | | | | | | | | W | S | | | | | | | | |
| | 8.30 | Bipy-3 | | | | | | | | | | | | | | S | W | | | | |
| | 7.56 | Bipy-4x+y | | | | | | | | | | | | | S | | S | W | | | |
| | 7.10 | Bipy-5 x+y | | | | | | | | | | | | | W | S | | S | | | |
| | 7.78 | Bipy-6x+y | | | | | | | | | | | | | | W | S | | | | |

## Table S-2: Folic Acid/Folic Acid COSY Crosspeaks

| COSY/FA | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Shift | Assignment | | | | | | | | | | | | | | | | | |
| Shift | | | 2.32 | 2.04 | 1.90 | 4.34 | 8.15 | 7.52 | 5.90 | 6.23 | 6.08 | 3.90 | 3.87 | 3.23 | 3.20 | 8.66 | 8.64 | | |
| Assignment | | | FA-22 | FA-21i | FA-21ii | FA-19 | FA-18 | FA-13/15 | FA-12/16 | FA-10x | FA-10y | FA-9ix | FA-9iy | FA-9iix | FA-9iiy | FA-7x | FA-7y | | |
| | 2.32 | FA-22 | | S | S | | | | | | | | | | | | | | |
| | 2.04 | FA-21i | S | | S | S | | | | | | | | | | | | | |
| | 1.90 | FA-21ii | S | S | | S | | | | | | | | | | | | | |
| | 4.34 | FA-19 | | S | S | | S | | | | | | | | | | | | |
| | 8.15 | FA-18 | | | | S | | | | | | | | | | | | | |
| | 7.52 | FA-13/15 | | | | | | | S | | | | | | | | | | |
| | 5.90 | FA-12/16 | | | | | | S | | | | | | | | | | | |
| | 6.23 | FA-10x | | | | | | | | | | S | | S | | | | | |
| | 6.08 | FA-10y | | | | | | | | | | | S | | S | | | | |
| | 3.90 | FA-9ix | | | | | | | | S | | | | S | | | | | |
| | 3.87 | FA-9iy | | | | | | | | | S | | | | S | | | | |
| | 3.23 | FA-9iix | | | | | | | | S | | S | | | | | | | |
| | 3.20 | FA-9iiy | | | | | | | | | S | | S | | | | | | |
| | 8.66 | FA-7x | | | | | | | | | | | | | | | | | |
| | 8.64 | FA-7y | | | | | | | | | | | | | | | | | |

## Table S-3: Bipyridine/Bipyridine NOESY Crosspeaks

| | Shift | Assignment | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Shift | | | 8.83 | 8.24 | 7.65 | 8.32 | 8.73 | 8.02 | 7.39 | 7.38 | 8.71 | 8.27 | 7.80 | 8.44 | 8.30 | 7.56 | 7.52 | 7.10 | 7.08 | 7.78 | 7.76 |
| Assignment | | | Bipy-3 | Bipy-4 | Bipy-5 | Bipy-6 | Bipy-3 | Bipy-4 | Bipy-5 | Bipy-6 | Bipy-3 | Bipy-4 | Bipy-5 | Bipy-6 | Bipy-3 | Bipy-4y | Bipy-4x | Bipy-5y | Bipy-5x | Bipy-6y | Bipy-6x |
| | 8.83 | Bipy-3 | | S | M | v.W | S | S | | | | | | | | | | | | v.W | |
| | 8.24 | Bipy-4 | S | | S | | S | | | | | | | | | | | | | | |
| | 7.65 | Bipy-5 | M | S | | S | | | | | | | | | | | | v.W | | v.W | |
| | 8.32 | Bipy-6 | v.W | | S | | | | | | ? | | | | | | | | | M | |
| | 8.73 | Bipy-3 | S | S | | | | S | M | | | | | | | W | | | | | |
| | 8.02 | Bipy-4 | S | | | | S | | S? | S? | | | | | | | | | | | |
| | 7.39 | Bipy-5 | | | | | M | S? | | ? | M? | | W? | S? | | | | | | | |
| | 7.38 | Bipy-6 | | | | | | S? | ? | | M? | | W? | S? | | | | | | | |
| | 8.71 | Bipy-3 | | | | ? | | | M? | M? | | S | W | | S? | W? | W | | | | |
| | 8.27 | Bipy-4 | | | | | | | | | S | | S | M | | S | | | | | |
| | 7.80 | Bipy-5 | | | | | | | W? | W? | W | S | | S | S | | | S | | | |
| | 8.44 | Bipy-6 | | | | | | | S? | S? | | M | S | | | | | | | | |
| | 8.30 | Bipy-3 | | | | | | | | | S? | | S | | | | | | | | |
| | 7.56 | Bipy-4y | | | | | W | | | | W? | S | | | | | | S | | | |
| | 7.52 | Bipy-4x | | | | | | | | | W | | | | | | | | S | | |
| | 7.10 | Bipy-5y | | | v.W | | | | | | | | S | | | S | | | | S | |
| | 7.08 | Bipy-5x | | | | | | | | | | | | | | | S | | | | S |
| | 7.78 | Bipy-6y | v.W | | v.W | M | | | | | | | | | | | | S | | | |
| | 7.76 | Bipy-6x | | | | | | | | | | | | | | | | | S | | |

## Table S-4: Folic Acid/Folic Acid NOESY Crosspeaks

| | Shift | Assignment | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Shift | | | 2.32 | 2.04 | 1.90 | 4.34 | 8.15 | 7.52 | 5.90 | 6.23 | 6.08 | 3.90 | 3.87 | 3.23 | 3.20 | 8.66 | 8.64 | | | | |
| Assignment | | | FA-22 | FA-21i | FA-21ii | FA-19 | FA-18 | FA-13/15 | FA-12/16 | FA-10x | FA-10y | FA-9ix | FA-9iy | FA-9iix | FA-9iiy | FA-7x | FA-7y | | | | |
| | 2.32 | FA-22 | | S | S | S | S | M | | | | | | | | | | | | | |
| | 2.04 | FA-21i | S | | S | S | S | v.W | | | | | | | | | | | | | |
| | 1.90 | FA-21ii | S | S | | S | S | M | | | | | | | | | | | | | |
| | 4.34 | FA-19 | S | S | S | | S | M | | | | | | | | | | | | | |
| | 8.15 | FA-18 | S | S | S | S | | S | M | | | | | | | | | | | | |
| | 7.52 | FA-13/15 | M | v.W | M | M | S | | S | W | M | M | M | M | M | | | | | | |
| | 5.90 | FA-12/16 | | | | | M | S | | S | S | S | S | S | S | M | M | | | | |
| | 6.23 | FA-10x | | | | | | W | S | | | M | | M | | M | | | | | |
| | 6.08 | FA-10y | | | | | | M | S | | | | M | | M | | S | | | | |
| | 3.90 | FA-9ix | | | | | | M | S | M | | | | S | | S | | | | | |
| | 3.87 | FA-9iy | | | | | | M | S | | M | | | | S | | S | | | | |
| | 3.23 | FA-9iix | | | | | | M | S | M | | S | | | | S | | | | | |
| | 3.20 | FA-9iiy | | | | | | M | S | | M | | S | | | | S | | | | |
| | 8.66 | FA-7x | | | | | | | M | M | | S | | S | | | | | | | |
| | 8.64 | FA-7y | | | | | | | M | | S | | S | | S | | | | | | |

## Table S-5: Folic Acid/Bipyridine NOESY Crosspeaks

| | Shift | Assignment | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Shift | | | 8.83 | 8.24 | 7.65 | 8.32 | 8.73 | 8.02 | 7.39 | 7.38 | 8.71 | 8.27 | 7.80 | 8.44 | 8.30 | 7.56 | 7.52 | 7.10 | 7.08 | 7.78 | 7.76 |
| Assignment | | | Bipy-3 | Bipy-4 | Bipy-5 | Bipy-6 | Bipy-3 | Bipy-4 | Bipy-5 | Bipy-6 | Bipy-3 | Bipy-4 | Bipy-5 | Bipy-6 | Bipy-3 | Bipy-4y | Bipy-4x | Bipy-5y | Bipy-5x | Bipy-6y | Bipy-6x |
| | 2.32 | FA-22 | | | | | | | | | | | | | | | | | | | |
| | 2.04 | FA-21i | | | | | | | | | | | | | | | | | | | |
| | 1.90 | FA-21ii | | | | | | | | | | | | | | | | | | | |
| | 4.34 | FA-19 | | | | | | | | | | | | | | | | | | | |
| | 8.15 | FA-18 | | | | | | | | | | | | | | | | | | | |
| | 7.52 | FA-13/15 | | | | | | | | | | S? | W? | | S? | | | | | W? | |
| | 5.90 | FA-12/16 | | | | M | | | | | M | W? | M | | W? | | | W | | M | |
| | 6.23 | FA-10x | | | | W | | | | | | | | | | | | | | | |
| | 6.08 | FA-10y | | | | M | | | | | M | M? | W | | M? | | | | | W | |
| | 3.90 | FA-9ix | | | | | | | | | | | | | | | | | | | |
| | 3.87 | FA-9iy | | | | M | | | | | | | | | | | | | | M | |
| | 3.23 | FA-9iix | | | | | | | | | | | | | | | | | | | |
| | 3.20 | FA-9iiy | | | | M | | | | | | | | | | | | | | M | |
| | 8.66 | FA-7x | | | | | | | | | | | | | | | | | | | |
| | 8.64 | FA-7y | | | | W? | | | | | | W? | | | W? | | | | | | |

## Table S-6: Bipyridine/Bipyridine HMBC Crosspeaks

| HMBC/BIPY | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Shift | Assignment | | | | | | | | | | | | | | | | | | |
| Shift | | | 8.83 | 8.24 | 7.65 | 8.32 | 8.73 | 8.02 | 7.39 | 7.38 | 8.71 | 8.27 | 7.80 | 8.44 | 8.30 | 7.56 | 7.52 | 7.10 | 7.08 | 7.78 | 7.76 |
| Assignment | | | Bipy-3 | Bipy-4 | Bipy-5 | Bipy-6 | Bipy-3 | Bipy-4 | Bipy-5 | Bipy-6 | Bipy-3 | Bipy-4 | Bipy-5 | Bipy-6 | Bipy-3 | Bipy-4y | Bipy-4x | Bipy-5y | Bipy-5x | Bipy-6y | Bipy-6x |
| | 157.0 | Bipy-2 | S | S | | | S | | | | | | | | | | | | | | |
| | 124.1 | Bipy-3 | | M | S | W | | | | | | | | | | | | | | | |
| | 137.8 | Bipy-4 | | | | S | | | | | | | | | | | | | | | |
| | 127.8 | Bipy-5 | S | | | S | | | | | | | | | | | | | | | |
| | 153.1 | Bipy-6 | | | | | | | | | | | | | | | | | | | |
| | 157.5 | Bipy-2 | S | | | | | S | S? | S? | | | | | | | | | | | |
| | 123.6 | Bipy-3 | M? | | | | | | M? | M? | | | | | | | | | | | |
| | 137.7 | Bipy-4 | | | | | | | | S | | | | | | | | | | | |
| | 127.3 | Bipy-5 | | | | | S | | | S? | | | | | | | | | | | |
| | 151.1 | Bipy-6 | | | | | | S | S? | | | | | | | | | | | | |
| | 157.3 | Bipy-2 | | | | | | | | | S | S | | S | M? | | | | | | |
| | 123.8 | Bipy-3 | | | | | | | | | | | S | | M? | | | | | | |
| | 137.7 | Bipy-4 | | | | | | | | | | | | S | | | | | | | |
| | 127.5 | Bipy-5 | | | | | | | | | S | | | M | | | | | | | |
| | 150.5 | Bipy-6 | | | | | | | | | | S | M | | | | M? | | | | |
| | 158.5 | Bipy-2 | | | | | | | | | S | M | | | M | S | M | | | S | S |
| | 123.6 | Bipy-3x/3y | | | | | | | | | | | | | | | | M | M | | |
| | 135.8 | Bipy-4y | | | | | | | | | | | | | | | | | | M | |
| | 135.8 | Bipy-4x | | | | | | | | | | | | | | | | | | | M |
| | 126.8 | Bipy-5x | | | | | | | | | | | | | M | | | | | | |
| | 126.8 | Bipy-5y | | | | | | | | | | | | | M | | | | | | |
| | 152.8 | Bipy-6x | | | | | | | | | | | | | | | M | | M | | |
| | 152.8 | Bipy-6y | | | | | | | | | | | | | | M | | M | | | |