Supplementary Material (ESI) for Dalton Transactions

Supplementary Materials

Altering pyridinone *N*-substituents to optimise activity as potential prodrugs for Alzheimer's disease

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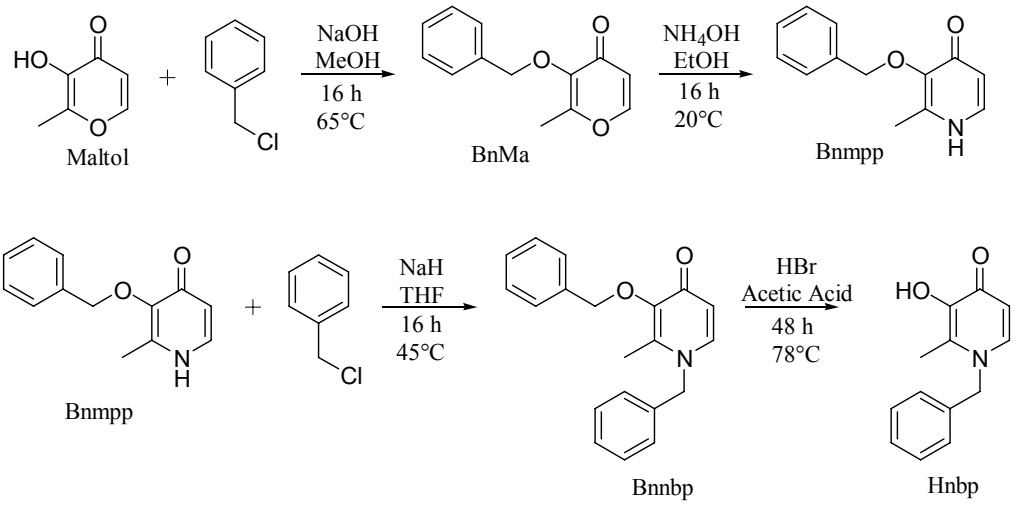
Experimental Details

^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker 300 MHz Varian XL-300 spectrometer; spectra were calibrated using residual solvent peaks. Mass spectra were obtained using a Bruker Esquire Ion Trap ESI-MS spectrometer. Elemental analysis for C, H and N was completed by Mr. David Wong at UBC Chemistry Mass Spectrometry and Microanalysis Services. X-ray diffraction data were collected and processed by Dr. B.O. Patrick at UBC Crystallography Services using a Bruker X8 APEX II diffractometer with graphite monochromated Mo-Kα radiation and the structure was solved using the Bruker SAINT software package.

3-Hydroxy-2-methyl-1-phenyl-4(1*H*)-pyridinone, Hppp

Prepared by a previously published method[1]
(Found: C, 71.66; H, 5.56; N, 6.96. Calc. for $C_{12}H_{11}NO_2$: C, 71.63; H, 5.51; N, 6.96%). δ_H(300 MHz; d_6-DMSO) 1.94 (3 H, s, 2-Me), 6.18 (1 H, d, J_{65} 9, 5-H), 7.43 (1 H, d, J_{56} 9, 6-H), 7.53 (5 H, m, Ph). δ_C(75 MHz; CD_3OD) 13.92 (2-Me), 104.49 (C5), 112.69 (C2), 128.10 (C_x), 131.00 (C_z), 131.21 (C_y), 133.06 (C6), 139.44 (C3), 143.34 (C_w), 171.68 (C4).

Scheme S1: Synthesis of **Hnbp**.



3-Benzyloxy-2-methyl-4(1*H*)-pyrone, BnMa

Prepared by a previously published method.[2] δ_H(300 MHz; $CDCl_3$): 2.10 (3 H, s, 2-Me), 5.16 (2 H, s, PhCH_2), 6.42 (1 H, d, J_{65} 5.6, 5-H), 7.33 (5 H, m, Ph), 7.61 (1 H, d, J_{56} 5.6, 6-H).

3-Benzyloxy-2-methyl-4(1*H*)-pyridinone, Bnmpp

Prepared by a previously published method.[2] δ_H(300 MHz; d_6-DMSO) 2.04 (3 H, s, 2-Me), 5.04 (2 H, s, CH_2Ph), 6.10 (1 H, d, 5-H), 7.35 (6 H, m, Ph and 6-H), 11.2 (1 H, s, 1-H).

1-Benzyl-3-benzyloxy-2-methyl-4(1*H*)-pyridinone, Bnnbp
Based on a previously published procedure.[3] Sodium hydride (0.29 g, 1.21 mmol) was added to a suspension of **Bnmpp** (2.00 g, 9.3 mmol) in THF (30 mL). Benzyl chloride (1.39 mL, 12.1 mmol) was added dropwise and the reaction was stirred for 16 h at 45 °C. Reaction progress was monitored with TLC (9:1 EtOAc:MeOH). Upon completion, the reaction mixture was diluted with EtOAc (20 mL) and washed with 5% NaCl solution, and with water. The organic phase was dried over $MgSO_4$. Solvents were removed under reduced pressure and the product was crystallised from water. Crystals of **Bnnbp** (1.54 g, 54%) were collected via suction filtration, washed with cold methanol and dried *in vacuo*. δ_H(300 MHz; d_6-DMSO) 1.95 (3 H, s, 2-Me), 5.05 (2 H, s, *O*-CH_2Ph), 5.17 (2 H, s, *N*-CH_2Ph), 6.22 (1 H, d, J_{65} 7.4, 5-H), 6.94 (2 H, d, *N*-CH_2Ph[H_x]), 7.32 (8H, m, *O*-CH_2*Ph*, *N*-CH_2Ph[H_{yz}]) 7.74 (1 H, d, J_{56} 7.4, 6-H).

1-Benzyl-3-hydroxy-2-methyl-4(1*H*)-pyridinone, Hnbp
Bnnbp (0.500 g, 1.63 mmol) was dissolved in 33 % HBr acetic acid (5 mL). The solution was refluxed for 48 h, allowed to cool and quenched with ether (5 mL). Excess HBr and acetic acid were removed under reduced pressure. The resulting solid was dissolved in water, brought to pH 12 using aqueous NaOH (5 M), and washed with EtOAc. The pH was adjusted to 6 using aqueous HCl (6 M) where the product precipitated. Colourless **Hnbp** (0.284 g, 81%) was collected via suction filtration and recrystallised from chloroform; crystals suitable for x-ray diffraction were obtained from chloroform. (Found: C, 72.16; H, 6.08; N, 6.43. Calc. for $C_{13}H_{13}NO_2$: C, 72.54; H, 6.09; N, 6.51%). δ_H(300 MHz; d_6-DMSO) 2.12 (3 H, s, 2-Me), 5.24 (2 H, s, *N*-CH_2Ph), 6.19 (1 H, d, J_{65} 7.2, 5-H), 7.07 (2 H, d, *N*-CH_2Ph[H_x]), 7.33 (3 H, m, *N*-CH_2Ph[H_{yz}]), 7.75 (1 H, d, J_{56} 7.3, 6-H). δ_C(75 MHz; CD_3OD) 12.30 (2-Me), 58.61 (*C*H_2Ph), 104.49 (C5), 112.80 (2-C), 127.38 (C_x), 129.36 (C_z), 130.35 (C_y), 133.34 (C6), 137.52 (C_w), 140.00 (C3), 171.17 (C4). **Crystal data.** $C_{13}H_{13}NO_2.CHCl_3$, M = 334.61, monoclinic, a = 11.7981(13), b = 10.8093(13), c = 12.5786(15) Å**,** U = 1527.7(3) Å^3, T = 173(2) K, space group $P2_1/c$ (no. 14), Z = 16,003 reflections measured, 3665 unique (R_{int} = 0.025) which were used in all calculations. The final $wR(F_2)$ was 0.110 (all data). CCDC entry 697418.

Bis(1-benzyl-2-methyl-3-oxy-4(1*H*)-pyridinato)copper(II), Cu(nbp)$_2$
Triethylamine (37.1 µL, 0.270 µmol) was added to a solution of **Hnbp** (0.0594 g, 0.277 mmol) in 13 mL 1:9 methanol:dichloromethane. This solution was added (dropwise) to $Cu(ClO_4)_2.6H_2O$ (0.050 g, 0.135 mmol) in 4 mL 1:9 methanol:dichloromethane. The reaction mixture was stirred at room temperature for 6 h yielding a green **Cu(nbp)$_2$**, which was isolated via suction filtration and crystallised via liquid-liquid diffusion from a mixture of ether and chloroform. Structure was confirmed by x-ray crystallography, and infrared spectroscopy. (Found: C, 63.30; H, 4.91; N, 5.69. Calc. for $C_{26}H_{24}CuN_2O_4$: C, 63.30; H, 4.92; N, 5.69%). **Crystal data.** $C_{26}H_{24}N_2O_4Cu.2CHCl_3$, M = 730.75, monoclinic, a = 6.3840(3), b = 21.0223(12), c = 11.7249(7) Å**,** U = 1562.53(15) Å^3, T = 173(2) K, space group $P\,2_1/n$ (no. 14), Z = 13,415 reflections measured, 3545 unique (R_{int} = 0.033) which were used in all calculations. The final $wR(F_2)$ was 0.163 (all data). CCDC entry 697416.

Bis(1-phenyl-2-methyl-3-oxy-4(1*H*)-pyridinato)copper(II), Cu(ppp)$_2$
Synthesised and crystallised in the same manner as Cu**(nbp)$_2$**. Structure confirmed by x-ray crystallography and infrared spectroscopy. (Found: C, 61.96; H, 4.36; N, 6.02. Calc for $C_{24}H_{20}CuN_2O_4$: C, 62.13; H, 4.34; N, 6.04%). **Crystal data.** $C_{24}H_{20}N_2O_4Cu.2CHCl_3$, M = 702.70, ortho-rhombic, a = 11.5466(11), b =11.6975(11), c = 20.9936(18) Å**,** U = 2835.5(5) Å^3, T = 173(2) K, space group *P bca* (no. 61), Z = 35,879 reflections measured, 2789 unique (R_{int} = 0.046) which were used in all calculations. The final $wR(F_2)$ was = 0.071 (all data). CCDC entry 697417.

Turbidity Assay

4-(2-Hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) buffer was prepared by diluting HEPES (4.76 g, 20.0 mmol) and NaCl (8.76 g, 150 mmol) with water (~850 mL) to nearly full volume (full volume is 1000 mL). Chelex® resin (a strong transition metal ion binding agent that is insolubilised on polystyrene) was added to the solution to rid the buffer of any residual transition metal ions that may confound results. The Chelex®-containing buffer solution was incubated in the refrigerator for 16 h. The buffer was allowed to warm to room temperature before adjusting the pH with 1 M NaOH (to pH 6.6 for Cu and pH 7.4 for Zn) and finally diluting to 1000 mL with deionised water. Separate buffers were prepared for the Cu- and Zn-mediated experiments, but both contained the same amount of HEPES, NaCl and Chelex®. Chelex® was removed via filtration through a Millipore 0.22 μm acetate filter which also ensured minimal contamination by other solids.

Human amyloid-beta peptide ($A\beta_{40}$, 1 mg) was carefully weighed, diluted with water (1.155 mL) then sonicated for one minute, allowed to stand for thirty seconds then sonicated for another minute. The Aβ was then filtered through a Whatman 0.2 μm nylon syringe filter. Diethylenetriaminepentaacetic acid (DTPA, 7.9 mg), **Hppp** (4.0 mg), and **Hnbp** (4.3 mg) were dissolved in HEPES buffer (100 mL, pH 6.6 for Cu(II) assay, pH 7.4 for Cu(II) assay). Zn(II) (200 μM, in pH 7.4 HEPES) and Cu(II) (200 μM, in pH 6.6 HEPES) solutions were prepared by diluting Aldrich Atomic Absorption Standard Solutions (Zn: 996 μg/mL in HCl 0.9 wt % (1.305 mL); Cu: 986 μg/mL in HNO_3 1 wt % (1.289 mL)).

A clear polystyrene Falcon® flat-bottomed 96-well plate was used to take the absorbance readings of the test solutions. Each well was loaded to a total volume of 200 μL (150 μL pyridinone proligand, 25 μL Aβ and 25 μL of metal solution) and done in quadruplicate to allow for statistical analysis of error. The DTPA positive control was done such that there was equivalent coordination sites of proligand to metal (i.e. DTPA wells contained 50 μL DTPA, 25 μL Aβ, 25 μL metal solution and 100 μL HEPES buffer). Positive and negative controls were used to ensure the validity of the results. To ensure that the test solutions were not responsible for the observed absorbance at 405 nm, blank readings were taken with all well components except Aβ peptide; it was noted that these solutions did not have significant absorbances at 405 nm.

The 96-well plate was loaded into a Molecular Devices Thermomax microplate reader programmed to agitate the plate for 20 seconds before measuring the absorbance of each well at 405 nm. The absorbance values obtained from the test wells (that

contained Aβ) were normalised with respect to the appropriate blank solutions (*i.e.* absorbance for **Hnbp** control, which contained 25 μL of buffer in place of the 25 μL Aβ, was subtracted from the **Hnbp** test condition which contained 25 μL of Aβ). The amount of absorbance is indicative of the amount of aggregated Aβ in the well, which is inversely proportional to the efficacy of our compounds. (More absorbance is due to more aggregated Aβ, which in pro-ligand tests mean that our compounds dissolved less of the aggregated Aβ.)

Trolox Equivalent Antioxidant Capacity (TEAC) Antioxidant Assay

The antioxidant capacity of **Hnbp** and **Hppp** were determined by a TEAC assay using Trolox and α-tocopherol (vitamin E) as standards. TEAC values were calculated according to an improved ABTS radical cation decolourization assay.[14] 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid diammonium salt (ABTS; 0.0387 g, 7.05 μmol, 7 mM) was dissolved in water (10 mL) and exposed to potassium persulphate (0.0066 g, 24.4 μmol, 2.45 mM). After a 16-hour incubation (dark, room temperature), the resulting solution was diluted with HPLC-grade methanol such that the absorbance of the solution at 745 nm was 0.7 ± 0.2 (2.5 mL diluted to final volume of 200 mL). Both the $ABTS^{\bullet+}$ solution and the test solutions were maintained at 30°C using a Fisher Scientific 1016D Isotemp water bath. $ABTS^{\bullet+}$ solution (2 mL) was loaded into a cuvette which was carefully placed into the light path of an HP 8543 UV-visible spectrophotometer. The absorbance was recorded at 745 nm prior to addition of the test solutions and then at times 1, 3 and 6 minutes after addition of the test solutions. Test solutions were prepared such that the addition of 20 μL to the radical would provide a reduction in absorbance by 20-80 %; this resulted in solutions ranging in concentration from 0.25-16 μM. Upon addition of 20 μL of test solution to the cuvette, the contents were mixed thoroughly by vigorously pipetting the mixture for twenty seconds. It was noted that consistency in the mixing process was very important; variations in mixing technique resulted in large error values. A new blank spectrum (methanol) was collected before each sample run.

Data were plotted such that for each time point a linear system of concentration versus absorbance was obtained. The slope of each plot was normalised with respect to that of Trolox to give the Trolox equivalence value for each time point. The error in each slope was calculated using linear regression techniques, and then statistically carried through to the final values for the TEAC. Compounds and standards were checked to ensure that they did not absorb at 745 nm.

Table S1: TEAC values for **Hppp**, **Hnbp** and α-tocopherol at six-minute time point.

Test Compound	6-min TEAC value	Error
Hppp	1.22	0.06
Hnbp	1.25	0.09
α-Tocopherol	1.0	0.1

MTT Assay

Media was removed from the culture flask via pipette, without affecting the human hepatocytes (cell line: HepG2) affixed to the bottom of the flask. Trypsin (~ 4 mL) was added to the culture flask, to release the cells from the wall of the dish. The cells were incubated (37°C) for 5 minutes, after which 10% fetal bovine serum (FBS) media (~ 4 mL) was added to quench the trypsin. The cells were suspended in solution and centrifuged for 3 minutes at 800 rpm. The cells were diluted to a concentration of 10^4 cells per 100 µL (10^4 cells or 100 µL per well). Cells were allotted to the wells and incubated for 24 h. Test compounds were dissolved in 10% FBS media (some requiring 1% DMSO due to insolubility) in non-sterile conditions, then filtered (to sterilise) through a 0.2 µM "Milex" polyethersulphone syringe filter. The initial solutions were diluted with 10% FBS media (with 1% DMSO for the less soluble compounds) to cover a range of concentrations from 2 to 2000 µM (to achieve well concentrations of 1 to 1000 µM). The cells were exposed to 100 µL of the compound-containing solutions; negative controls received 100 µL of 10% FBS media (with 1% DMSO for the DMSO controls). Cisplatin was used as a positive control for cell death using the same range of concentrations as the test compounds. Cells were then incubated with test compounds for 72 h. After incubation, 50 µL of 2.5 g/L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in phosphate buffered saline was added and cells were incubated for an additional 3 h. Supernatant was removed from the wells by suction through a 21-G needle attached to a vacuum pump and the cells were dissolved in 150 µL DMSO. Absorbance was measured at 577 nm and referenced to the control and blank wells to find the relative cell viabilities for each compound and concentration: each concentration of each compound was done in quadruplicate such that appropriate statistical analysis could be completed.

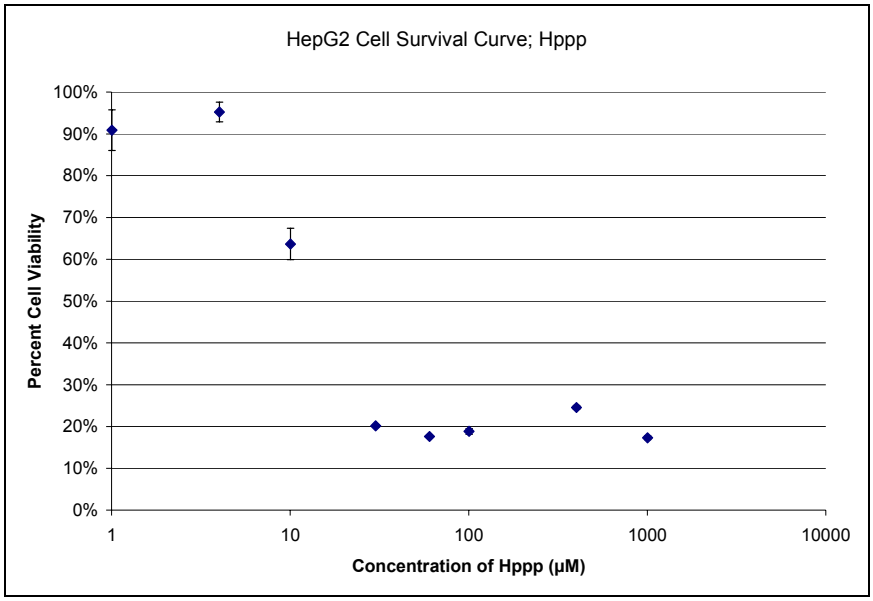


Figure S1: Sample survival plot of HepG2 cells exposed to varying concentrations of **Hppp** for 72 h, as monitored by MTT assay.

X-Ray Diffraction Data

X-ray data was collected and processed by Dr. B.O. Patrick at UBC using a Bruker X8 APEX II diffractometer with graphite monochromated Mo-Kα radiation; the structure was solved using Bruker SAINT software package (SAINT. Version 7.46A. Bruker AXS Inc., Madison, Wisconsin, USA. 1997-2007). All C-H hydrogen atoms in all three structures were placed in calculated positions, only the hydroxyl hydrogen in **Hnbp** was treated differently, in this case the hydrogen was located in a difference map and refined isotropically. In Cu(**nbp**)$_2$ the benzyl substituent is disordered and was modeled in two orientations with equivalent populations.

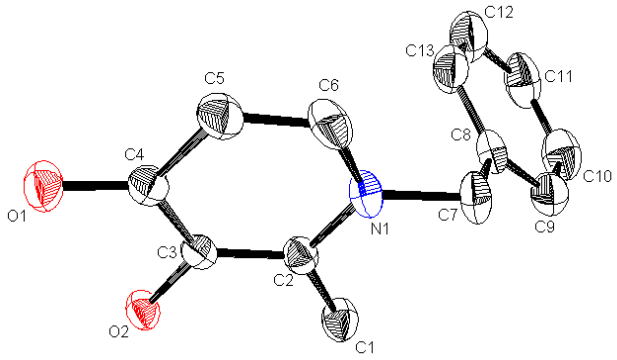


Figure S2: Ellipsoid plot (50% probability, H-atoms removed for clarity) of **Hnbp**.

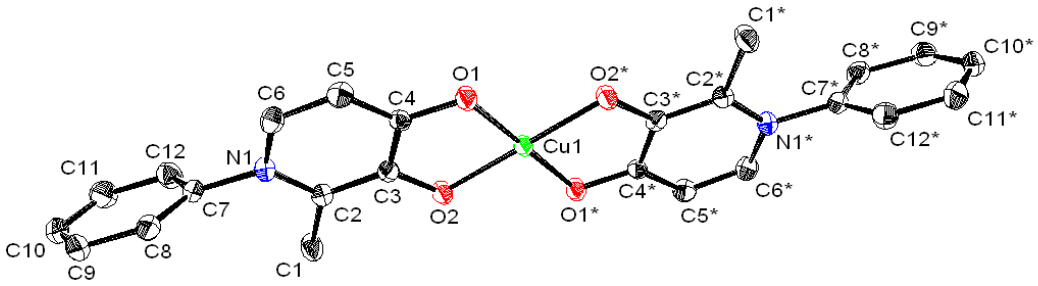


Figure S3: Ellipsoid plot (50% probability, H-atoms removed for clarity) of Cu(**ppp**)$_2$.

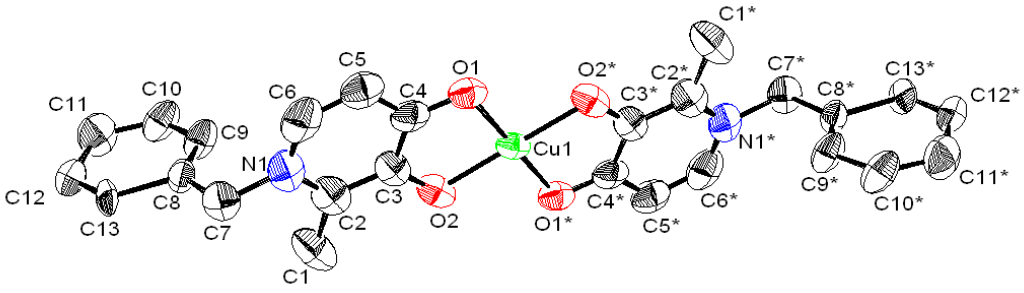


Figure S4: Ellipsoid plot (50% probability, H-atoms removed for clarity) of Cu(**nbp**)$_2$.

Table S2: Selected crystal data for **Hnbp** pro-ligand.

crystal data	**Hnbp•CHCl$_3$**
formula	$C_{13}H_{13}NO_2.CHCl_3$
fw	334.61
crystal system, space group	monoclinic, *P* $2_1/c$ (#14)
***a* (Å)**	11.7981(13)
***b* (Å)**	10.8093(13)
***c* (Å)**	12.5786(15)
α (deg)	90.0
β (deg)	107.760(6)
γ (deg)	90.0
***V* [Å^3]**	1527.7(3)
***Z*, D_{calcd} (g/cm^3)**	4, 1.455
***μ*(Mo Kα), (cm^{-1})**	5.99
F_{000}	688.00
temp. (K)	173(2)
reflns collcd / unique	16 003/3665
	(R_{int} = 0.025)
residuals (*F*^{2}, all data)	wR_2 = 0.110
residuals (*F*, *I* > 2σ(*I*))	R_1 = 0.045
crystal colour, habit	colourless, plate

Table S3: Selected crystal data for Cu(**ppp**)$_2$ and Cu(**nbp**)$_2$ complexes.

crystal data	**Cu(ppp)$_2$• 2CHCl$_3$**	**Cu(nbp)$_2$• 2CHCl$_3$**
formula	$C_{24}H_{20}N_2O_4Cu.2CHCl_3$	$C_{26}H_{24}N_2O_4Cu.2CHCl_3$
fw	702.70	730.75
crystal system, space group	orthorhombic, *P bca* (#61)	monoclinic, *P* $2_1/n$ (#14)
***a* (Å)**	11.5466(11)	6.3840(3)
***b* (Å)**	11.6975(11)	21.0223(12)
***c* (Å)**	20.9936(18)	11.7249(7)
α (deg)	90.0	90.0
β (deg)	90.0	96.787(2)
γ (deg)	90.0	90.0
***V* [Å^3]**	2835.5(5)	1562.53(15)
***Z*, D_{calcd} (g/cm^3)**	4, 1.646	2, 1.553
μ(Mo Kα), (cm^{-1})	13.73	12.49
F_{000}	1420.00	742.00
temp. (K)	173(2)	173(2)
reflns collcd / unique	35 879/2789	13 415/3545
	(R_{int} = 0.046)	(R_{int} = 0.033)
residuals (*F*^{2}, all data)	wR_2 = 0.071	wR_2 = 0.139
residuals (*F*, *I* > 2σ(*I*))	R_1 = 0.028	R_1 = 0.053
crystal colour, habit	green, prism	green, prism

References:

[1] Z. Zhang, S. J. Rettig and C. Orvig, *Inorg. Chem.*, 1991, **30**, 509-515.
[2] C. A. Barta, K. Sachs-Barrable, J. Jia, K. H. Thompson, K. M. Wasan and C. Orvig, *Dalton Trans.*, 2007, **43**, 5019-5030.
[3] H. Kitagawa, K. Kumura, S. Takahata, M. Iida and K. Atsumi, *Bioorg. Med. Chem.*, 2006, **15**, 1106-1116.
[4] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans, *Free Radical Biol. Med.*, 1999, **26**, 1231-1237.