Electronic Supplementary Material (ESI) for Chemical Communications

Supporting information

Maleimide-functionalised Organoruthenium Anticancer Agents and their Binding to Thiol-containing Biomolecules

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Materials and methods

All reactions were carried out in dry solvents under an inert atmosphere. All chemicals were obtained from commercial suppliers and were of analytical grade. [Ru(η^6-*N*-benzylmaleimide)Cl_2]$_2$,[1] 1,3,5-triaza-7-phosphatricyclo[3.3.1.1]decane (PTA),[2] 3,5,6-bicyclophosphite-1,2-*O*-isopropylidene-α-D-glucofuranoside and 3,5,6-bicyclophosphite-1,2-*O*-cyclohexylidene-α-D-glucofuranoside[3] were synthesised using literature procedures. ^{1}H, ^{13}C{^{1}H} and ^{31}P{^{1}H} NMR spectra were recorded at 25 °C on a Bruker FT NMR spectrometer Avance III 500 MHz at 500.10 (^{1}H), 125.75 (^{13}C{^{1}H}) and 202.44 MHz (^{31}P{^{1}H}) or on a Bruker DPX 400 MHz instrument at 400.13 (^{1}H), 100.63 (^{13}C{^{1}H}) and 161.98 MHz (^{31}P{^{1}H}). 2D NMR spectra were collected in a gradient-enhanced mode. The temperature dependence of the ^{31}P{^{1}H} NMR of **1** was recorded in D_2O (Aldrich) containing 100 mM NaCl (Fluka) and 2.5% of d_7-DMF and was studied from 20–50 °C in 10 °C increments. Melting points were measured on a Büchi B-540 apparatus and are uncorrected. Elemental analysis was determined by the Laboratory for Elemental Analysis, Faculty of Chemistry, University of Vienna, on a Perkin–Elmer 2400 CHN Elemental Analyzer. Electrospray ionisation mass spectra were recorded on a Bruker esquire$_{3000}$.
X-ray diffraction measurements of **1** were performed on a Bruker X8 APEX II CCD diffractometer at 100 K. The crystal was positioned at 35 mm from the detector and 2621 frames for 20 sec over 1° scan width were measured. The data were processed using the SAINT software package.[4] Crystal data, data collection parameters, and structure refinement details are given in Table S1. The structure was solved by direct methods and refined by full-matrix least-squares techniques. Non-hydrogen atoms were refined with anisotropic displacement parameters. H atoms were inserted at calculated positions and refined with a riding model. The molecule of co-crystallised methanol was found disordered over two positions with s.o.f. 50:50. The following computer programs were used: structure solution, SHELXS-97; refinement, SHELXL-97;[5] molecular diagrams, ORTEP-3.[6] Crystallographic data for the structural analysis of **3** has been deposited with the Cambridge Crystallographic Data Centre, CCDC No. 837010. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Synthesis of complexes 1–4

Dichlorido(η^6-*N*-benzylmaleimide)(1,3,5-triaza-7-phosphatricyclo[3.3.1.1]decane)ruthenium(II) (1)

A solution of [Ru(η^6-*N*-benzylmaleimide)Cl_2]$_2$ (144 mg, 0.2 mmol) and 1,3,5-triaza-7-phosphatricyclo[3.3.1.1]decane (PTA), (64 mg, 0.4 mmol) in dry DMF (10 mL) was stirred for 3 h at room temperature. The solvent was reduced *in vacuo* to about 5 mL, diethyl ether (25 mL) was added and the reaction mixture was kept at 4 °C for two days. Long red needles were isolated by filtration, washed with diethyl ether (3 × 5 mL) and dried under vacuum.

Yield: 193 mg (92%); m.p. > 300 °C (decomp.); Elemental analysis, found % C, 38.80; H, 4.23; N, 10.43. calcd. for $C_{17}H_{21}N_4O_2Cl_2PRu \cdot 0.5H_2O$, C, 38.87; H, 4.22; N, 10.67. MS (ESI^+): *m/z* 481.0 $[M - Cl]^+$, 540.8 $[M + Na]^+$. ^{1}H NMR (500.10 MHz, DMSO-d_6, 25 °C): δ = 7.08 (s, 2 H, CH(CO)), 5.89 (t, J = 5.0 Hz, 2 H, H-Ar), 5.79 (d, J = 6.0 Hz, 2 H, H-Ar), 5.39 (t, J = 5.0 Hz, 1 H, H-Ar), 4.46 (s, 6 H, NCH_2N), 4.28 (s, 2 H, Ar-CH_2N), 4.22 (s, 6 H, PTA-PCH_2N) ppm. $^{13}C\{^1H\}$ NMR (125.75 MHz, DMSO-d_6, 25 °C): δ = 178.1 (CH(*C*O)), 171.0 (*C*H(CO)), 135.2 (C-Ar), 129.1 (C-Ar), 127.7 (C-Ar), 88.8 (C-Ar), 86.7 (C-Ar), 80.4 (C-Ar), 72.6 (NCH_2N), 52.4 (PCH_2N), 28.6 ($ArCH_2N$) ppm. $^{31}P\{^1H\}$ NMR (202.44 MHz, DMSO-d_6, 25 °C): δ = -31.0 ppm.

Dichlorido(η^6-*N*-benzylmaleimide)(3,5,6-bicyclophosphite-1,2-*O*-isopropylidene-α-D-glucofuranoside)ruthenium(II) (2)

A suspension of [Ru(η^6-*N*-benzylmaleimide)Cl_2]$_2$ (72 mg, 0.1 mmol) and 1,2-*O*-isopropylidene-D-glucofuranose 3,5,6-bicyclophosphite (50 mg, 0.2 mmol) in CH_2Cl_2 (20 mL) was stirred for 5 h at 40 °C. The solvent was reduced *in vacuo* to about 3 mL and diethyl ether (25 mL) was added. The orange precipitate was filtered, washed with diethyl ether (3 × 5 mL) and dried under vacuum.

Yield: 119 mg (92%); m.p. 161-163 °C (decomp.); Elemental analysis, found % C, 38.04; H, 3.70; N, 2.19. calcd. for $C_{20}H_{22}NO_8Cl_2PRu \cdot 0.5CH_2Cl_2$, C, 37.89; H, 3.57; N, 2.16. MS (ESI^+): *m/z* 572.0 $[M - Cl]^+$, 629.7 $[M + Na]^+$. ^{1}H NMR (500.10 MHz, $CDCl_3$, 25 °C): δ = 6.77 (s, 2 H, CH(CO)), 6.24 (s, 1 H, H-1), 6.00-6.05 (m, 2 H, H-Ar), 5.91 (d, J = 5.0 Hz, 2 H, H-Ar), 5.73 (brs, 1 H, H-Ar), 5.13 (m, 1 H, H-5), 4.84 (brs, 1 H, H-3), 4.80 (brs, 1 H, H-2), 4.47-4.54 (m, 3 H, H-6, NCH_2), 4.33 (m, 2 H, H-6', H-4), 1.53 (s, 3 H, $C(CH_3)_2$), 1.38 (s, 3 H, $C(CH_3)_2$) ppm.

$^{13}C\{^{1}H\}$ NMR (125.75 MHz, $CDCl_3$, 25 °C): δ = 176.5 (CH(*C*O)), 169.8 (CH(*C*O)), 134.5 (*C*H(CO)), 112.7 (*C*$(CH_3)_2$), 105.7 (C-1), 99.1 (C-Ar), 93.6 (C-Ar), 92.2 (C-Ar), 90.6 (C-Ar), 90.4 (C-Ar), 88.8 (C-Ar), 83.7 (J = 7.3 Hz, C-2), 79.4 (J = 8.2 Hz, C-3), 76.8 (C-4), 74.9 (J = 5.2 Hz, C-5), 69.3 (J = 9.0 Hz, C-6), 40.0 (NCH_2), 26.9 (C(*C*$H_3)_2$), 26.2 (C(*C*$H_3)_2$) ppm. $^{31}P\{^{1}H\}$ NMR (202.44 MHz, $CDCl_3$, 25 °C): δ = 131.8 ppm.

Dichlorido(η^6-*N*-benzylmaleimide)(3,5,6-bicyclophosphite-1,2-*O*-cyclohexylidene-α-D-glucofuranoside)ruthenium(II) (3)

A suspension of [Ru(η^6-*N*-benzylmaleimide)$Cl_2]_2$ (72 mg, 0.1 mmol) and 1,2-*O*-cyclohexylidene-D-glucofuranose 3,5,6-bicyclophosphite (58 mg, 0.2 mmol) in CH_2Cl_2 (20 mL) was stirred for 5 h at 40 °C. The solvent was reduced *in vacuo* to about 5 mL and diethyl ether (25 mL) was added. The orange precipitate was filtered, washed with diethyl ether (3 × 5 mL) and dried under vacuum.

Yield: 127 mg (98%); m.p. 205-206 °C (decomp.); Elemental analysis, found % C, 41.69; H, 4.11; N, 2.03. calcd. for $C_{23}H_{26}NO_8Cl_2PRu \cdot 0.25CH_2Cl_2$, C, 41.76; H, 3.99; N, 2.10. MS (ESI^+): *m/z* 611.8 $[M - Cl]^+$, 671.8 $[M + Na]^+$. ^1H NMR (500.10 MHz, $CDCl_3$, 25 °C): δ = 6.78 (s, 2H, CH(CO)), 6.25 (brs, 1 H, H-1), 5.99-6.06 (m, 2 H, H-Ar), 5.91 (d, J = 5.0 Hz, 2 H, H-Ar), 5.74 (brs, 1 H, H-Ar), 5.14 (m, 1 H, H-5), 4.85 (brs, 1 H, H-3), 4.80 (brs, 1 H, H-2), 4.50-4.55 (m, 3 H, H-6, NCH_2), 4.34 (m, 2 H, H-6', H-4), 1.68-1.71 (m, 4 H, C_6H_{10}), 1.43-1.58 (m, 6 H, C_6H_{10} ppm. $^{13}C\{^{1}H\}$ NMR (125.75 MHz, $CDCl_3$, 25 °C): δ = 176.6 (CH(*C*O)), 169.8 (CH(*C*O)), 134.6 (*C*H(CO)), 113.3 (*C*$(CH_3)_2$), 105.4 (C-1), 99.1 (C-Ar), 93.6 (C-Ar), 92.2 (C-Ar), 90.7 (C-Ar), 90.5 (C-Ar), 88.8 (C-Ar), 83.3 (J = 6.4 Hz, C-2), 79.6 (J = 8.0 Hz, C-3), 75.5 (C-4), 75.0 (C-5), 69.5 (C-6), 40.0 (NCH_2), 36.5 (C_6H_{10}), 35.7 (C_6H_{10}), 24.8 (C_6H_{10}), 23.8 (C_6H_{10}), 23.5 (C_6H_{10}) ppm. $^{31}P\{^{1}H\}$ NMR (202.44 MHz, $CDCl_3$, 25 °C): δ = 131.9 ppm.

Dichlorido(η^6-*N*-benzylmaleimide)(triphenylphosphine)ruthenium(II) (4)

A solution of [Ru(η^6-*N*-benzylmaleimide)$Cl_2]_2$ (144 mg, 0.2 mmol) and triphenylphosphine (105 mg, 0.4 mmol) in dry DMF (10 mL) was stirred for 3 h at room temperature. The solvent was reduced *in vacuo* to about 5 mL, diethyl ether (25 mL) was added and the reaction mixture was kept at 4 °C for two days. The red, micro-crystalline product was filtered, washed with diethyl ether (3 × 5 mL) and dried under vacuum.

Yield: 235 mg (94%); m.p. 202-203 °C (decomp.); Elemental analysis, found % C, 54.58; H, 4.64; N, 3.96. calcd. for $C_{29}H_{24}NO_2Cl_2PRu \cdot 0.5H_2O \cdot 0.75DMF$, C, 54.77; H, 4.45; N, 3.58. MS (ESI^+): *m/z* 585.8 $[M - Cl]^+$, 621.8 $[M + H]^+$. ^1H NMR (500.10 MHz, DMSO-d_6, 25 °C): δ = 7.62-7.66 (m, 6 H, PPh_3), 7.44-7.49 (m, 9 H, PPh_3), 7.05 (s, 2 H, CH(CO)), 5.55-5.56 (m, 2 H, Ar-H), 5.36-5.39 (m, 2 H, Ar-H), 5.07 (m, 1 H, Ar-H), 4.31 (s, 2 H, N-CH_2) ppm. $^{13}C\{^1H\}$ NMR (125.75 MHz, DMSO-d_6, 25 °C): δ = 177.8 (CH(*C*O)), 171.0 (*C*H(CO)), 134.4 (C-Ar), 130.8 (C-Ar), 128.5 (C-Ar), 101.0 (C-Ar), 90.2 (C-Ar), 90.0 (C-Ar), 82.4 (C-Ar), 28.6 ($ArCH_2N$) ppm. $^{31}P\{^1H\}$ NMR (202.44 MHz, DMSO-d_6, 25 °C): δ = 27.9 ppm.

Physiochemical Studies

For the thiol-binding studies, solutions of **1** and **2** (1 mM) and Cys·HCl·H_2O, Met (both Fluka), *N*-acetylcysteine, glutathione (both Sigma) or *N*-acetylcysteine methyl ester (Fluka) at a molar ratio of 1 : 1.2 were prepared in D_2O, D_2O containing 100 mM NaCl or phosphate buffer [prepared from $Na_2HPO_4·7H_2O$ (Merck) and $NaH_2PO_4·2H_2O$ (Fluka) with D_2O (20 mM, pD 7.4, 100 mM NaCl)]. The reaction of **1** with HSA (2 : 1; Sigma) in D_2O was followed by ^{1}H NMR spectroscopy. The reaction mixtures were analyzed several times after the start of the incubation at room temperature (compare figure captions).

nESI–FT-ICR-MS. For electrospray ionisation mass spectrometry, the samples were placed into a 96-well plate in an AdvionTriVersa™ robot (Advion Biosciences, Ithaca NY) equipped with a 5.5 µm-nozzle chip. The ESI robot was controlled with ChipSoft v7.2.0 software and the following parameters were used for positive ion mode: gas pressure, 0.45 psi; voltage, 1.7 kV; sample volume, 7.00 µl. The samples were analyzed in positive ion mode using a hyphenated linear ion-trap–FT-ICR mass spectrometer which consisted of an LTQ XL and a 12 T FT-ICR MS (both Thermo Scientific, Bremen, Germany). The mass spectra were calibrated externally with the Thermo Scientific calibration solution and the peaks were assigned with an accuracy of normally < 1 ppm. The Xcalibur software bundle was utilised for recording (Tune Plus version 2.2 SP1; Thermo Scientific, Bremen, Germany) and data analysis (Qual Browser version 2.2; Thermo Scientific, Bremen, Germany). All the given *m/z* values correspond to the most abundant isotopic peak of each isotopic cluster.

The samples contained the complexes and Cys, *N*-acetylcysteine or glutathione in a molar ratio of 1 : 1 in water (50 µM). The reaction mixtures were kept at room temperature for several hours (for exact measurement sequence compare figure captions) and were diluted 1 : 50 with water or water/methanol (80/20) prior to analysis.

SEC-UV/vis and -ICP-MS. For binding studies of **1** with albumin and human serum, a Superdex 200 SEC column (GE Healthcare, 0.8 ml/min ammonium acetate buffer 100 mM, pH 7.4) was used to separate unreacted **1** from its macromolecular binding partners. For SEC-UV/vis studies, samples containing equimolar amounts of **1** and HSA (both 50 µM) were prepared in PBS

(10 mM, 104 mM NaCl, pH 7.4; Fluka) and analyzed immediately after mixing and after 45 min reaction time. A ThermoFinnigan Surveyor liquid chromatography system was used with 10 µL injection volume and the UV/vis detector was set to record the signals at 200, 254 and 280 nm.

For SEC-ICP-MS studies, a Perkin Elmer Series 200 liquid chromatography system was hyphenated to a Perkin Elmer ICP-MS DRC II. The ICP-MS system was operated in no gas mode at 1100 W, with nebuliser and auxiliary gas flows of 0.90 and 1.30 l/min, respectively. The instrument was optimised prior to analysis with the Elan 6100 DRC calibration solution. The samples were introduced *via* a Meinhard nebuliser and a cyclonic spray chamber. The separation conditions were used as described for the LC-UV/vis studies with 20 µL injection from a solution prepared by diluting reaction mixtures of 50 µM HSA (Sigma) and **1** (molar ratio 1 : 1; in 10 mM PBS) by 1 : 10 with water or of **1** (250 µM ; in 5 mM PBS; equimolar to HSA in human serum) and human serum (Sigma) by 1 : 50 with water. The ICP-MS was set to record the ^{102}Ru and ^{195}Pt traces (the latter as internal standard for the spray/instrument stability). The reaction mixtures were analyzed several times over a time period of 3 h and after 72 h.

Cytotoxicity in cancer cell lines

Cell lines and culture conditions. CH1 cells originate from an ascites sample of a patient with a papillary cystadenocarcinoma of the ovary and were a generous gift from Lloyd R. Kelland, CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, UK. SW480 (adenocarcinoma of the colon, human), and A549 (non-small cell lung cancer, human) cells were kindly provided by Brigitte Marian (Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Austria). All cell culture reagents were obtained from Sigma-Aldrich Austria. Cells were grown in 75 cm^2 culture flasks (Iwaki) as adherent monolayer cultures in Eagle's Minimal Essential Medium (MEM) supplemented with 10% heat-inactivated foetal calf serum, 1 mM sodium pyruvate and 2 mM L-glutamine. Cultures were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2.

MTT assay conditions. Cytotoxicity was determined by the colorimetric MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide, purchased from Fluka) microculture assay. For this purpose, cells were harvested from culture flasks by trypsinisation and seeded in 100 µL aliquots MEM supplemented with 10% heat-inactivated foetal calf serum, 1 mM sodium pyruvate, 4 mM L-glutamine and 1% non-essential amino acids (100×) into 96-well microculture plates (Iwaki). Cell densities of 1.5×10^3 cells/well (CH1), 2.5×10^3 cells/well (SW480) and 4×10^3 cells/well (A549) were chosen in order to ensure exponential growth of untreated controls throughout the experiment. Cells were allowed to settle and resume exponential growth for 24 h. The test compounds were dissolved and serially diluted in the same medium and added in 100 µL aliquots to the microcultures (if necessary due to limited solubility, the maximum concentration tested was added in 200 µL aliquots after removal of the medium), and cells were exposed to the test compounds for 96 hours. At the end of the exposure period, all media were replaced by 100 µL/well RPMI1640 culture medium (supplemented with 10% heat-inactivated foetal calf serum) plus 20 µL/well MTT solution in phosphate-buffered saline (5 mg/ml). After incubation for 4 h, the supernatants were removed, and the formazan crystals formed by vital cells were dissolved in 150 µL DMSO per well. Optical densities at 550 nm were measured with a microplate reader (Tecan Spectra Classic), using a reference wavelength of 690 nm to correct for unspecific absorption. The quantity of vital cells was expressed in terms of T/C values by

comparison to untreated control microcultures, and 50% inhibitory concentrations (IC_{50}) were calculated from concentration-effect curves by interpolation. Evaluation is based on means from at least three independent experiments, each comprising at least three replicates per concentration level.

Figures and Tables

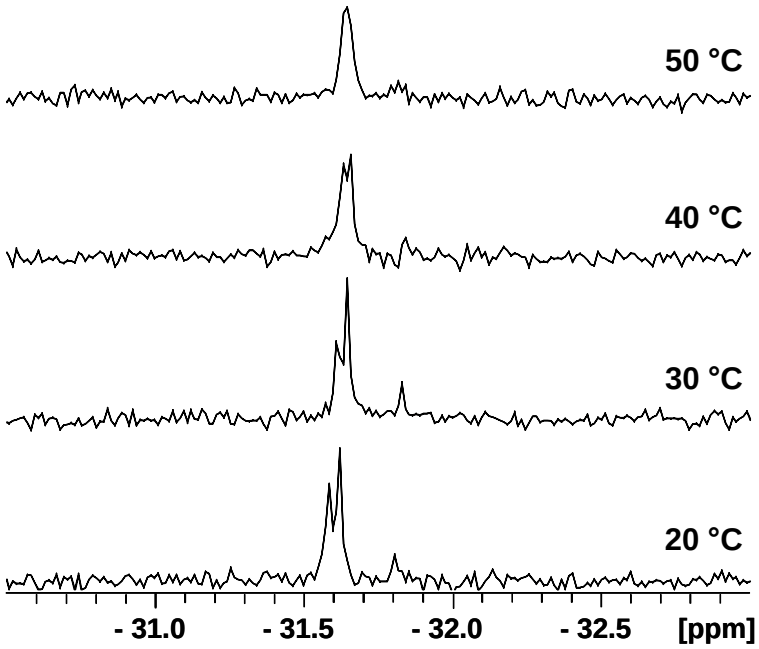

Figure S1. Effect of the temperature on the $^{31}P\{^{1}H\}$ NMR spectrum of **1**.

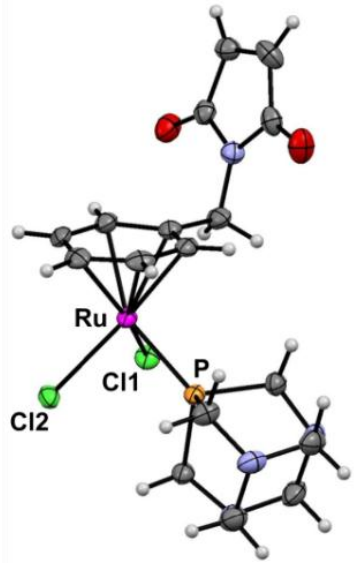

Figure S2. Molecular structure of compound 1 (selected bond lengths and angles are given in Table S2).

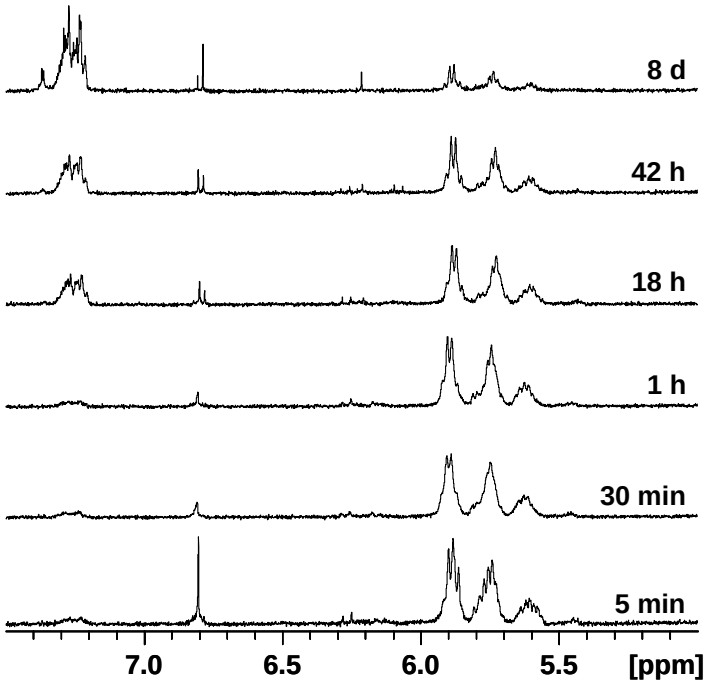

Figure S3. ^{1}H NMR spectroscopic study on the reaction of **1** with Cys (1 : 1) in D_2O/100 mM NaCl followed for 8 days.

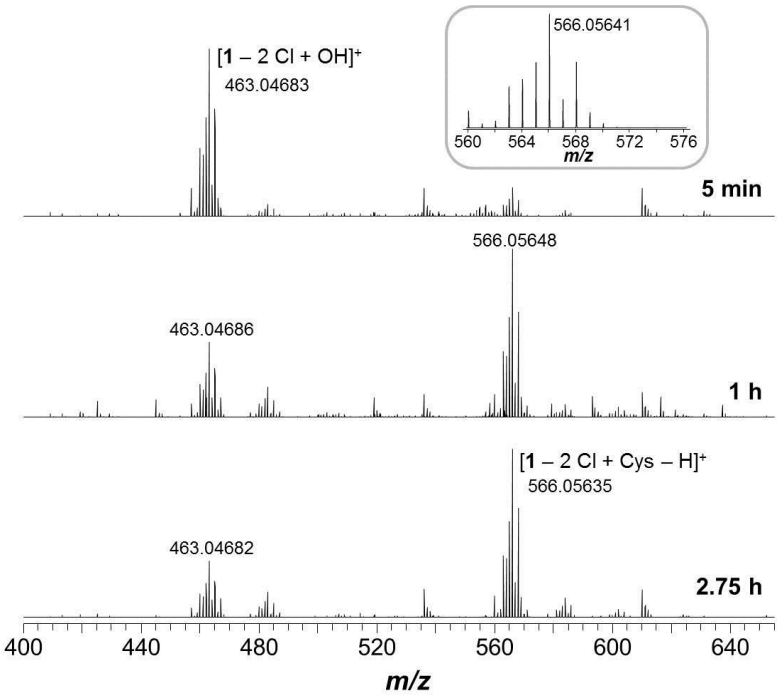

Figure S4. Time course of the reaction of **1** with Cys in H_2O, as investigated by ESI-FT-ICR-MS. The inset shows the calculated isotope pattern and the accurate mass/charge ratio of the most abundant isotopic peak of the Cys adduct.

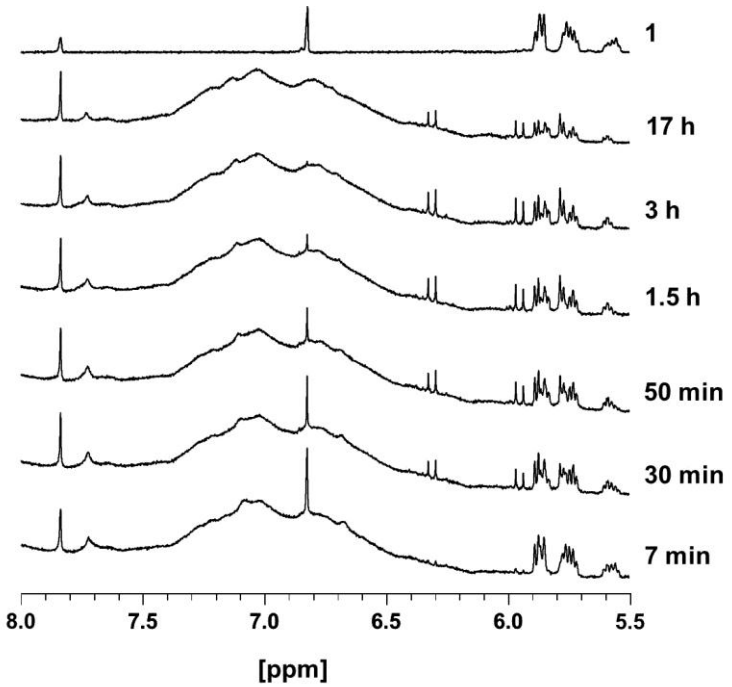

Figure S5. Time course of the reaction of **1** with HSA (1 : 2) in D_2O/100 mM NaCl relative to the signal of d_7-DMF as internal standard (the sample of **1** contained only half the amount of d_7-DMF than the protein-containing samples).

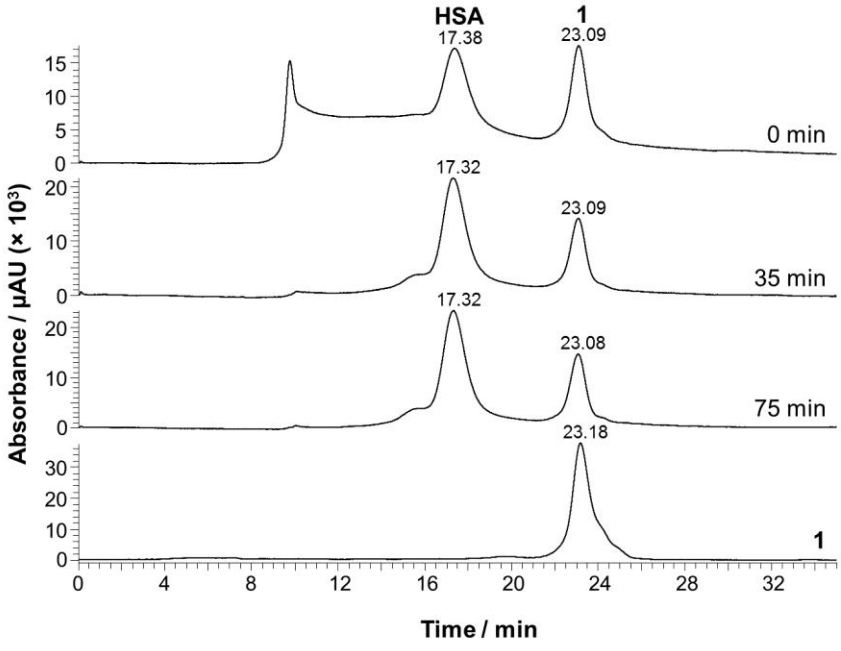

Figure S6. SEC chromatograms recorded with a spectrophotometric detector at 254 nm for the reaction of **1** with human serum albumin in PBS. The peak at *ca.* 10 min is an artefact.

Table S1. Crystal data and structure refinement for **1**.

Empirical formula	$C_{18}H_{25}Cl_2N_4O_3PRu$
Formula weight	548.36
Temperature / K	100(2)
Crystal system	monoclinic
Space group	$P2_1/_c$
a / Å	14.2423(7)
b / Å	11.6629(6)
c / Å	13.1191(6)
β / °	99.589(2)
Volume / Å^3	2148.72(18)
Z	4
ρ_{calc} / mg mm^{-3}	1.695
μ / mm^{-1}	1.081
F(000)	1112
Crystal size / mm^3	0.98 × 0.50 × 0.07
2Θ range for data collection	4.54 to 56°
Index ranges	$-18 \le h \le 18$, $-15 \le k \le 15$, $-17 \le l \le 17$
Reflections collected	107474
Independent reflections	5194 [R(int) = 0.0702]
Data/restraints/parameters	5194/2/271
Goodness-of-fit on F^2	1.017
Final R indexes [I > 2σ (I)]	R_1 = 0.0310
Final R indexes [all data]	wR_2 = 0.0866
Largest diff. peak / hole / e Å^{-3}	0.633/-0.499

Table S2. Selected bond lengths (Å) and angles (°) for **1** as compared to RAPTA-C.

	1	**RAPTA-C** [a]
Ru-Cl1	2.4071(6)	2.412(3), 2.426(3)
Ru-Cl2	2.4242(6)	2.429(2), 2.425(3)
Ru-P	2.3010(6)	2.296(2), 2.298(3)
Ru-arene$_{centroid}$	1.692	1.702, 1.701
P-Ru-Cl1	82.28(2)	87.09(9), 85.29(10)
P-Ru-Cl2	84.66(2)	83.42(8), 82.78(9)
Cl1-Ru-Cl2	88.36(2)	87.25(8), 94.0(3)
Cl1-Ru-arene$_{centroid}$	127.36	126.85, 127.23
Cl2-Ru-arene$_{centroid}$	128.24	127.86, 127.78
P-Ru-arene$_{centroid}$	130.37	129.60, 129.76

[a] The molecular structure of RAPTA-C contains two crystallographically independent molecules in the unit cell; taken from ref. 7.

Table S3. Calculated *m/z* values for selected reaction products between **1** and Cys.

Experiment	**Species**	**calculated *m/z* value**	**experimental *m/z* value**	**Δ / ppm**
MS	[**1** – 2Cl + OH]$^+$	463.04722	463.04862	3.0
	[**1** + Cys – 2 Cl – H]$^+$	566.05641	566.05635	0.1
MS^2	[**1** + Cys – 2 Cl – pta – 2H]$^+$	408.97937	408.97846	2.2
	[**1** + Cys – 2 Cl – $C_3H_5NO_2$]$^+$	479.02429	479.02212	4.5
MS^3	[**1** – 2 Cl – pta + S + 2H]$^+$	321.947256	321.94745	0.6
	[**1** + Cys – 2 Cl – pta – CO_2 – H]$^+$	364.98951	364.98917	0.9
	[**1** + Cys – 2 Cl – pta – O – 3H]$^+$	390.96880	390.96798	2.1

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