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Is the Reactivity of M(II)−Arene Complexes of 3-Hydroxy-2(1*H*)-pyridones to Biomolecules the Anticancer Activity Determining Parameter?

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Hydroxypyr(id)ones are versatile ligands for the synthesis of organometallic anticancer agents, equipping them with fine-tunable pharmacological properties. Herein, we report on the preparation, mode of action, and in vitro anticancer activity of Ru^{II}− and Os^{II}−arene complexes with alkoxycarbonylmethyl-3-hydroxy-2-pyridone ligands. The hydrolysis and binding to amino acids proceed quickly, as characterized by NMR spectroscopy and ESI mass spectrometry. However, the reaction with amino acids causes cleavage of the pyridone ligands from the metal center because the amino acids act as multidentate ligands. A similar behavior was also observed during the reactions with the model proteins ubiquitin and cytochrome c, yielding mainly [protein + M(η^6-*p*-cymene)] adducts (M = Ru, Os). Notably the ligand cleavage of the Os derivative was significantly slower than of its Ru analogue, which could explain its higher activity in in vitro anticancer assays. Furthermore, the reaction of the compounds to 5′-GMP was characterized and coordination to the *N7* of the guanine moiety was demonstrated by ^{1}H NMR spectroscopy and X-ray diffraction analysis. CDK2/Cyclin A protein kinase inhibition studies revealed potent activity of the Ru and Os complexes.

Introduction

Ruthenium-based coordination compounds and organometallics are promising antitumor agents, and NAMI-A and KP1019 have passed clinical phase I trials.[1−6] Ruthenium complexes benefit from low ligand exchange rates (similar to platinum complexes), low systemic toxicity, a range of oxidation states accessible under physiological conditions, some physicochemical properties similar to iron, and affinity to serum transport proteins.[7−10] In recent years, the anticancer activity of Ru drug candidates has often been compared to that of their Os analogues; however, no clear-cut structure−activity relationship could be derived. Organometallic ruthenium(II)−arene and also their analogous osmium(II)−arene compounds provide an option to fine-tune the chemical reactivity and also pharmacological properties such as water solubility, oral bioavailability, stability in plasma, pharmacokinetic behavior, etc., on the basis of the choice of ligands and coordination geometry.[5]

One of the first concepts applied in the quest for organometallic Ru anticancer drugs was to combine metal−arene moieties with bioactive ligands as in the first organometallic ruthenium species tested for anticancer activity, i.e., [Ru(η^6-C_6H_6)Cl_2(metronidazole)], containing the common antiinfective agent metronidazole.[11] This strategy was also successful with a variety of enzyme inhibitors such as paullones, ethacrynic acid, and staurosporine.[12−14] However, the combination of noncytotoxic ligands with metals also led to compounds with

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significant anticancer activity as demonstrated for pta, ethylenediamine, and many other examples.[4−6,15]

Metal−arene complexes of the type $[M(\eta^6\text{-arene})(XY)Cl]^{n+}$ (XY = a bidentate *N*,*N*-, *N*,*O*-, *O*,*O*- or *S*,*O*-chelating ligand; $n = 0, 1$) have shown promising cytotoxicity profiles. The coordination of simple bidentate *N*,*N* ligands such as ethylenediamine to organometallic Ru(II)−arene species resulted in compounds exhibiting high cytotoxicity in cancer cells, including cisplatin-resistant cell lines.[4] Monofunctional covalent binding accompanied by a hydrogen bond to DNA nucleobases is considered essential for the mode of action,[16] with the ultimate targets of bioorganometallics still remaining unclear and controversially discussed.[4,5,17] Complexes bearing paullone-derived *N*,*N*-chelating ligands are highly active in in vitro anticancer assays, regardless of whether ruthenium or osmium is the central metal.[13] The same metal centers equipped with *N*,*O*- and *O*,*O*-chelating ligands such as those derived from the amino acids glycine, L-alanine, and L-proline, or with 8-oxyquinolinato, tropolonato, or acetylacetonato give compounds which are moderately cytotoxic,[18] whereas with picolinato and picolinamide ligands promising in vitro activities, also in cisplatin-resistant cells, were observed.[19−21]

Mononuclear ruthenium−arene complexes with *O*,*O*-bound maltol-derived ligands are not or are only marginally cytotoxic.[22,23] Switching from *O*,*O*- to *S*,*O*-donor systems increases the anticancer activity in vitro,[24] probably because of higher stability in the presence of biomolecules.[25] Exchanging pyrones by pyridones (HPs), for example in dinuclear ruthenium−arene complexes with an adjustable spacer length, results in high anticancer activity with the potential to overcome drug resistance.[26−30] Another class of *O*,*O*-donor system ligands are hydroxypyr(id)ones, and their metal complexes have demonstrated potential in different research areas, including medicinal inorganic chemistry. They have been studied extensively as metal chelators,[31,32] magnetic resonance imaging (MRI) contrast media,[33−41] matrix metalloprotein inhibitors,[42−44] receptors for Li^+ or Na^+,[45−47] anthrax lethal factor inhibitors,[48,49] antidiabetic,[50−53] antiviral,[49] and antibacterial agents.[48,49,54] We have reported a series of mono- and polynuclear metal−arene complexes of 3-hydroxy-4-pyr(id)ones and investigated their tumor inhibiting properties.[23,24,26,27,29,55] In order to extend the structure−activity relationships, a series of mononuclear metal(II)−arene complexes of ruthenium and osmium with *O*,*O*-bound alkoxycarbonylmethyl-3-hydroxy-2(1*H*)-pyridones were synthesized and characterized with regard to stability toward hydrolysis, biological activity, and interaction with DNA model nucleobases such as 5′-GMP and 9-ethylguanine and with proteins in order to correlate affinity to biomolecules with anticancer activity.

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Experimental Section

Materials and Methods. All reactions were carried out in dry solvents under an inert atmosphere. Chemicals obtained from commercial suppliers were used as received and were of analytical grade; methanol and CH_2Cl_2 were dried using standard procedures. OsO_4 (99.8%; ***Caution!*** *OsO_4 is highly toxic and volatile!*[56]) and $RuCl_3 \cdot 3H_2O$ (40.4%) were purchased from Johnson Matthey; ubiquitin from bovine red blood cells and horse heart cytochrome c from Sigma; L-alanine and α-terpinene from Acros; glycine and L-histidine from Merck; L-cysteine, 5′-dGMP, 5′-GMP, and $N_2H_4 \cdot 2HCl$ from Fluka; and L-methionine from Sigma-Aldrich. The solvents for ESI-MS studies were methanol (VWR Int., HiPerSolv CHROMANORM), formic acid (Fluka), and Milli-Q H_2O (18.2 MΩ, Synergy 185 UV Ultrapure, Millipore, France). The dimers bis[dichlorido(η^6-*p*-cymene)ruthenium(II)],[57,58] bis[dibromido(η^6-*p*-cymene)ruthenium(II)],[28] bis[diiodido(η^6-*p*-cymene)ruthenium(II)],[28] and bis[dichlorido(η^6-*p*-cymene)osmium(II)],[59] and the ligands 1-[(ethoxycarbonyl)methyl]-3-hydroxy-2-(1*H*)-pyridone **a**,[60] 1-[(methoxycarbonyl)methyl]-3-hydroxy-2-(1*H*)-pyridone **b**,[61] and 1-ethoxycarbonylmethyl-3-hydroxy-4-methyl-2-(1*H*)-pyridone **c**[62] were synthesized using literature procedures.

^{1}H, ^{31}P{^{1}H}, and ^{13}C{^{1}H} NMR spectra were recorded at 25 °C on a Bruker FT NMR spectrometer Avance III 500 MHz at 500.10 (^{1}H), 202.44 (^{31}P{^{1}H}), and 125.75 MHz (^{13}C{^{1}H}), and 2D NMR data were collected in a gradient-enhanced mode. Melting points were measured on a Büchi B-540 apparatus and are uncorrected. Elemental analysis was done on a Perkin−Elmer 2400 CHN elemental analyzer by the Laboratory for Elemental Analysis, Faculty of Chemistry, University of Vienna. Electrospray ionization mass spectra were recorded on a Bruker esquire$_{3000}$ ion trap instrument.

X-ray diffraction measurements of single crystals of **1a**, **1b**, and **5a** were performed on a Bruker X8 APEX II CCD diffractometer at 100 K. The crystals were positioned at 35 mm from the detector and 1917, 1580, and 1446 frames for 30, 50, and 60 s over 1° were measured for **1a**, **1b**, and **5a**, respectively. The data were processed using the SAINT Plus software package.[63] Crystal data, data collection parameters, and structure refinement details are given in Table 1. The structures were 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 isopropyl group of the *p*-cymene moiety in **5a** was found disordered. The disorder was resolved with constrained anisotropic displacement parameters and restrained bond distances using EADP and SADI instructions of SHELX97, respectively. The site occupation factors were refined to about 0.60:0.40. The following software programs and tables were used: structure solution, SHELXS-97;[64,65] refinement, SHELXL-97;[65,66] and molecular diagrams, ORTEP-3.[67]

Synthesis. General Procedure. A solution of 3-hydroxy-2-(1*H*)-pyridone (2.2 equiv) and NaOMe (3.5 equiv) in 20 mL MeOH was stirred for about 30 min. A solution of [(η^6-cymene)MX(μ-X)]$_2$ (1.0 equiv; M = Ru, Os; X = Cl, Br, I) in 5 mL CH_2Cl_2 was added, and the mixture was stirred for 4 h at RT. The solvent was removed under reduced pressure. The residue was redissolved in CH_2Cl_2, and filtered to remove undissolved impurities. The filtrate was evaporated under vacuum. Recrystallization from CH_2Cl_2 and diethyl ether gave the pure product.

[Chlorido(η^6-*p*-cymene){*N*-[(ethoxycarbonyl)methyl]-3-oxo-κ*O*-2-(1*H*)-pyridonato-κ*O*}ruthenium(II)] (1a). The title compound was synthesized from *N*-[(ethoxycarbonyl)methyl]-3-hydroxy-2-(1*H*)-pyridone (130 mg, 0.33 mmol), NaOMe (38 mg, 0.70 mmol), and [(η^6-*p*-cymene)RuCl(μ-Cl)]$_2$ (92 mg, 0.15 mmol), following the general procedure. Crystals suitable for X-ray diffraction analysis were grown by slow diffusion of diethyl ether into a solution of **1a** in CH_2Cl_2. Yield: 130 mg (93%), mp 221−222 °C dec. Elemental analysis: found C, 48.55; H, 4.88; N, 2.94. Calcd for $C_{19}H_{24}O_4NClRu$: C, 48.87; H, 5.18; N, 2.99; MS (ESI$^+$) *m*/*z* 432.2 [M − Cl]$^+$. ^{1}H NMR (500.10 MHz, $CDCl_3$, 25 °C): δ 6.67 (dd, $^3J_{(H5,H6)}$ = 8 Hz, $^4J_{(H4,H6)}$ = 1 Hz, 1H, H-6$_{Py}$), 6.40 (dd, $^3J_{(H4,H5)}$ = 7 Hz, $^4J_{(H4,H6)}$ = 1 Hz, 1H, H-4$_{Py}$), 6.26 (dd, $^3J_{(H5,H6)}$ = 7 Hz, $^3J_{(H4,H5)}$ = 7 Hz, 1H, H-5$_{Py}$), 5.62 (d, $^3J_{(H,H)}$ = 6 Hz; 1H, Ar-H), 5.60 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar-H), 5.38 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar-H), 5.34 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar-H), 5.11 (d, $^2J_{gem(H,H)}$ = 17 Hz, 1H, NC*H*$_2$CO), 4.27 (m, 3H, NC*H*$_2$CO, OC*H*$_2$CH$_3$), 2.93 (m, 1H, C*H*(CH$_3$)$_2$), 2.31 (s, 3H, Ar-CH$_3$), 1.33 (m, 9H, (C*H*$_3$)$_2$CH-Ar, OCH$_2$C*H*$_3$) ppm. ^{13}C{^{1}H} NMR (125.75 MHz, $CDCl_3$, 25 °C): δ 167.0 (C-2), 166.3 (C-3), 160.9 (*C*OOEt), 121.1 (C-4), 117.6 (C-6), 112.7 (C-5), 99.7 (C-Ar), 94.4 (C-Ar), 81.4 (CH-Ar), 80.5 (CH-Ar), 79.3 (CH-Ar), 77.8 (CH-Ar), 61.9 (O*C*H$_2$CH$_3$), 51.3 (N*C*H$_2$CO), 31.2 (*C*H(CH$_3$)$_2$), 22.5 (CH(*C*H$_3$)$_2$), 22.4 (CH(*C*H$_3$)$_2$), 18.5 (Ar-*C*H$_3$), 14.2 (OCH$_2$*C*H$_3$) ppm.

[Chlorido(η^6-*p*-cymene){*N*-[(methoxycarbonyl)methyl]-3-oxo-κ*O*-2-(1*H*)-pyridonato-κ*O*}ruthenium(II)] (1b). The title compound was synthesized from *N*-[(methoxycarbonyl)methyl]-3-hydroxy-2-(1*H*)-pyridone (40 mg, 0.22 mmol), NaOMe (19 mg, 0.35 mmol) and [(η^6-*p*-cymene)RuCl(μ-Cl)]$_2$ (61 mg, 0.10 mmol), following the general procedure. Crystals suitable for X-ray diffraction analysis were grown by slow diffusion of diethyl ether into a solution of **1b** in CH_2Cl_2. Yield: 81 mg (90%), mp > 200 °C dec. Elemental analysis: found C, 47.39; H, 4.63; N, 2.98. Calcd for $C_{18}H_{22}NO_4ClRu$: C, 47.74; H, 4.89; N, 3.09; MS (ESI$^+$) *m*/*z* 418.0 [M − Cl]$^+$. ^{1}H NMR (500.10 MHz, $CDCl_3$, 25 °C): δ 6.67 (dd, $^3J_{(H5,H6)}$ = 8 Hz, $^4J_{(H4,H6)}$ = 1 Hz, 1H, H-6$_{Py}$), 6.40 (d, $^3J_{(H4,H5)}$ = 7 Hz, 1H, H-4$_{Py}$), 6.26 (dd, $^3J_{(H5,H6)}$ = 7 Hz, $^3J_{(H4,H5)}$ = 8 Hz, 1H, H-5$_{Py}$), 5.51 (d, $^3J_{(H,H)}$ = 6 Hz; 1H, Ar-H), 5.50 (d, $^3J_{(H,H)}$ = 6 Hz; 1H, Ar-H), 5.31 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar-H), 5.31 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar-H), 5.11 (d, $^2J_{gem(H,H)}$ = 17 Hz, 1H, NC*H*$_2$CO), 4.27 (d, $^2J_{gem(H,H)}$ = 17 Hz, 1H, NC*H*$_2$CO), 3.81 (s, 3H, OC*H*$_3$), 2.88 (m, 1H, C*H*(CH$_3$)$_2$), 2.29 (s, 3H, Ar-C*H*$_3$), 1.34 (d, $^3J_{(H,H)}$ = 6.9 Hz, 3H, C*H*(C*H*$_3$)$_2$), 1.30 (d, $^3J_{(H,H)}$ = 6.9 Hz, 3H, CH(C*H*$_3$)$_2$) ppm. ^{13}C{^{1}H} NMR (125.75 MHz, $CDCl_3$, 25 °C): δ 167.3 (C-2), 166.2 (C-3), 160.7 (*C*OOMe), 121.1 (C-4), 117.7 (C-6), 112.8 (C-5), 99.4 (C-Ar), 94.8 (C-Ar), 81.3 (CH-Ar), 80.5 (CH-Ar), 79.2 (CH-Ar), 77.3 (CH-Ar), 52.7 (COO*C*H$_3$), 51.3 (N*C*H$_2$CO), 31.1 (*C*H(CH$_3$)$_2$), 22.4 (CH(*C*H$_3$)$_2$), 22.3 (CH(*C*H$_3$)$_2$), 18.5 (Ar-CH$_3$) ppm.

[Chlorido(η^6-*p*-cymene){*N*-[(ethoxycarbonyl)methyl]-3-oxo-κ*O*-4-methyl-2(1*H*)-pyridonato-κ*O*}ruthenium(II)] (1c). The title compound was synthesized from *N*-[(ethoxycarbonyl)methyl]-3-hydroxy-4-methyl-2(1*H*)-pyridone (85 mg, 0.40 mmol), NaOMe (25 mg, 0.44 mmol), and [(η^6-*p*-cymene)RuCl(μ-Cl)]$_2$ (122 mg,

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Table 1. Crystal Data and Details of Data Collection for **1a**, **1b**, and **5a**[a]

compound	1a	1b	5a
chemical formula	$C_{19}H_{24}ClNO_4Ru$	$C_{18}H_{22}ClNO_4Ru$	$C_{27}H_{33}F_3N_6O_8Ru\,S$
M (g mol^{-1})	466.91	452.89	759.72
temperature (K)	100(2)	100(2)	100(2)
crystal size (mm)	0.25 × 0.20 × 0.13	0.14 × 0.10 × 0.04	0.20 × 0.13 × 0.13
crystal color, habit	orange, block	orange, block	orange, block
crystal system	triclinic	triclinic	triclinic
space group	$P\bar{1}$	$P\bar{1}$	$P\bar{1}$
a (Å)	9.5302(4)	9.4338(4)	11.6654(5)
b (Å)	10.1465(4)	10.0892(4)	12.5639(9)
c (Å)	10.5931(5)	10.2654(5)	12.6860(7)
V (Å^3)	960.63(7)	922.26(7)	1606.11(16)
Z	2	2	2
D_c (g cm^{-3})	1.614	1.631	1.571
μ (mm^{-1})	0.979	1.017	0.627
F(000)	476	460	776
Θ range (deg)	2.64 to 30.12	2.64 to 30.08	2.51 to 30.11
h range	−13/13	−13/13	−16/16
k range	−14/14	−14/14	−17/17
l range	−14/14	−14/14	−17/17
no. refls.	5610	5384	9411
no. parameters	237	227	425
R_{int}	0.0358	0.0736	0.0609
R_1 (obs.)	0.0241	0.0356	0.0366
wR_2 (all data)	0.0596	0.0802	0.0908
S	1.003	1.014	1.005

[a] Refinement was by full-matrix least-squares (F_o^2) for all reflections, $R_1 = \sum||F_o| - |F_c||/\sum|F_o|$, $wR_2 = \{\sum[w(F_o^2 - F_c^2)^2]/\sum[w(F_o^2)^2]\}^{1/2}$. Goodness of fit, $S = \{\sum[w(F_o^2 - F_c^2)^2]/(n-p)\}^{1/2}$.

0.20 mmol), following the general procedure. Yield: 110 mg (57%), mp >200 °C dec. Elemental analysis: found C, 49.60; H, 5.23; N, 2.89. Calcd for $C_{20}H_{26}NO_4ClRu$: C, 49.95; H, 5.45; N, 2.91; MS (ESI$^+$) m/z 446.0 [M − Cl]$^+$. ^{1}H NMR (500.10 MHz, $CDCl_3$, 25 °C): δ 6.34 (d, $^3J_{(H5,H6)}$ = 7 Hz, 1H, H-6$_{Py}$), 6.18 (dd, $^3J_{(H5,H6)}$ = 7 Hz, 1H, H-5$_{Py}$), 5.52 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar-H), 5.49 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar-H), 5.25 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar-H), 5.24 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar-H), 5.13 (d, $^2J_{gem(H,H)}$ = 17 Hz, 1H, NCH_2CO), 4.28 (m, 2H, OCH_2CH$_3$), 4.19 (d, $^2J_{gem(H,H)}$ = 17 Hz, 1H, NCH_2CO), 2.89 (m, 1H, CH(CH$_3$)$_2$) 2.31 (s, 3H, Ar-CH_3), 2.19 (s, 3H, Py-CH$_3$), 1.34 (m, 9H, CH(CH_3)$_2$, OCH$_2$CH_3) ppm. ^{13}C{^{1}H} NMR (125.75 MHz, $CDCl_3$, 25 °C): δ 167.2 (C-2), 164.3 (C-3), 158.2 (*C*OOEt), 128.9 (C-4), 120.0 (C-6), 115.4 (C-5), 98.5 (C-Ar), 95.8 (C-Ar), 81.3 (CH-Ar), 80.5 (CH-Ar), 77.7 (CH-Ar), 77.1 (CH-Ar), 61.8 (O*C*H$_2$CH$_3$), 51.1 (N*C*H$_2$CO), 31.1 (*C*H(CH$_3$)$_2$), 22.4 (CH(*C*H$_3$)$_2$), 22.3 (CH(*C*H$_3$)$_2$), 18.5 (Ar-*C*H$_3$), 15.5 (Py-*C*H$_3$), 14.2 (OCH$_2$*C*H$_3$) ppm.

[Bromido(η^6-*p*-cymene){*N*-[(ethoxycarbonyl)methyl]-3-oxo-κ*O*-2-(1*H*)-pyridonato-κ*O*}ruthenium(II)] (2a). The title compound was synthesized from *N*-[(ethoxycarbonyl)methyl]-3-hydroxy-2-(1*H*)-pyridone (43 mg, 0.22 mmol), NaOMe (19 mg, 0.35 mmol), and [(η^6-*p*-cymene)RuBr(μ-Br)]$_2$ (79 mg, 0.10 mmol), following the general procedure. Yield: 90 mg (88%), mp 204−205 °C dec. Elemental analysis: found C, 44.44; H, 4.69; N, 2,78. Calcd for $C_{19}H_{24}NO_4BrRu$: C, 44.63; H, 4.73; N, 2.74; MS (ESI$^+$) m/z 432.0 [M − Br]$^+$. ^{1}H NMR (500.10 MHz, $CDCl_3$, 25 °C): δ 6.67 (dd, $^3J_{(H5,H6)}$ = 8 Hz, $^4J_{(H4,H6)}$ = 1 Hz, 1H, H-6$_{Py}$), 6.40 (dd, $^3J_{(H4,H5)}$ = 7 Hz, $^4J_{(H4,H6)}$ = 1 Hz, 1H, H-4$_{Py}$), 6.26 (dd, $^3J_{(H5,H6)}$ = 7 Hz, $^3J_{(H4,H5)}$ = 7 Hz, 1H, H-5$_{Py}$), 5.53 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar-H), 5.52 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar-H), 5.32 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar-H), 5.31 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar-H), 5.11 (d, $^2J_{gem(H,H)}$ = 17 Hz, 1H, NCH_2CO), 4.27 (m, 3H, NCH_2CO, OCH_2CH$_3$), 2.93 (m, 1H, CH(CH$_3$)$_2$) 2.31 (s, 3H, Ar-CH$_3$), 1.33 (m, 9H, CH(CH_3)$_2$, OCH$_2$CH_3) ppm. ^{13}C{^{1}H} NMR (125.75 MHz, $CDCl_3$, 25 °C): δ 167.0 (C-2), 166.3 (C-3), 160.9 (*C*OOEt), 121.1 (C-4), 117.6 (C-6), 112.7 (C-5), 99.7 (C-Ar), 94.4 (C-Ar), 81.4 (CH-Ar), 80.5 (CH-Ar), 79.3 (CH-Ar), 77.8 (CH-Ar), 61.9 (O*C*H$_2$CH$_3$), 51.3 (N*C*H$_2$CO), 31.2 (*C*H(CH$_3$)$_2$), 22.5 (CH(*C*H$_3$)$_2$), 22.4 (CH(*C*H$_3$)$_2$), 18.5 (Ar-*C*H$_3$), 14.2 (OCH$_2$*C*H$_3$) ppm.

[(η^6-*p*-Cymene){*N*-[(ethoxycarbonyl)methyl]-3-oxo-κ*O*-2-(1*H*)-pyridonato-κ*O*}iodidoruthenium(II)] (3a). The title compound was synthesized from *N*-[(ethoxycarbonyl)methyl]-3-hydroxy-2-(1*H*)-pyridone (43 mg, 0.22 mmol), NaOMe (19 mg, 0.35 mmol), and [(η^6-*p*-cymene)RuI(μ-I)]$_2$ (98 mg, 0.10 mmol), following the general procedure. Yield: 69 mg (62%), mp 214−215 °C dec. Elemental analysis: found C, 40.53; H, 4.26; N, 2.5. Calcd for $C_{19}H_{24}NO_4IRu$: C, 40.87; H, 4.33; N, 2.51; MS (ESI$^+$) m/z 432.0 [M − I]$^+$. ^{1}H NMR (500.10 MHz, $CDCl_3$, 25 °C): δ 6.66 (dd, $^3J_{(H5,H6)}$ = 1 Hz, $^4J_{(H4,H6)}$ = 7 Hz, 1H, H-6$_{Py}$), 6.41 (dd, $^3J_{(H4,H5)}$ = 7 Hz, $^4J_{(H4,H6)}$ = 1 Hz, 1H, H-4$_{Py}$), 6.26 (dd, $^3J_{(H5,H6)}$ = 7 Hz, $^3J_{(H4,H5)}$ = 8 Hz, 1H, H-5$_{Py}$), 5.58 (d, $^3J_{(H,H)}$ = 5 Hz, 1H, Ar-H), 5.54 (d, $^3J_{(H,H)}$ = 5 Hz, 1H, Ar-H), 5.38 (d, $^3J_{(H,H)}$ = 5 Hz, 1H, Ar-H), 5.31 (d, $^3J_{(H,H)}$ = 5 Hz, 1H, Ar-H), 5.02 (d, $^2J_{gem(H,H)}$ = 17 Hz, 1H, NCH_2CO), 4.28 (m, 3H, NCH_2CO, OCH_2CH$_3$), 2.95 (m, 1H, CH(CH$_3$)$_2$) 2.33 (s, 3H, Ar-CH$_3$), 1.33 (m, 9H, CH(CH_3)$_2$, OCH$_2$-CH_3) ppm. ^{13}C{^{1}H} NMR (125.75 MHz, $CDCl_3$, 25 °C): δ 167.3 (C-2), 166.7 (C-3), 161.7 (*C*OOEt), 121.0 (C-4), 117.5 (C-6), 112.8 (C-5), 100.2 (C-Ar), 94.4 (C-Ar), 80.0 (CH-Ar), 79.4 (CH-Ar), 78.9 (CH-Ar), 77.0 (CH-Ar), 61.1 (O*C*H$_2$CH$_3$), 51.4 (N*C*H$_2$CO), 31.4 (*C*H(CH$_3$)$_2$), 22.6 (CH(*C*H$_3$)$_2$), 22.5 (CH(*C*H$_3$)$_2$), 19.2 (Ar-*C*H$_3$), 14.2 (OCH$_2$*C*H$_3$) ppm.

[Chlorido(η^6-*p*-cymene){*N*-[(ethoxycarbonyl)methyl]-3-oxo-κ*O*-2-(1*H*)-pyridonato-κ*O*}osmium(II)] (4a). The title compound was synthesized from *N*-[(ethoxycarbonyl)methyl]-3-hydroxy-2-(1*H*)-pyridone (44 mg, 0.22 mmol), NaOMe (19 mg, 0.35 mmol), and [(η^6-*p*-cymene)OsCl(μ-Cl)]$_2$ (79 mg, 0.10 mmol), following the general procedure. Yield: 85 mg (76%), mp 222−223 °C dec. Elemental analysis: found C, 40.81; H, 4.14; N, 2.50. Calcd for $C_{19}H_{24}O_4NClOs$: C, 41.04; H, 4.35; N, 2.52; MS (ESI$^+$) m/z 520.2 [M − Cl]$^+$. ^{1}H NMR (500.10 MHz, $CDCl_3$, 25 °C): δ 6.68 (dd, $^3J_{(H5,H6)}$ = 8 Hz, $^4J_{(H4,H6)}$ = 1 Hz, 1H, H-6$_{Py}$), 6.41 (dd, $^3J_{(H4,H5)}$ = 7 Hz, $^4J_{(H4,H6)}$ = 1 Hz, 1H, H-4$_{Py}$), 6.26 (dd, $^3J_{(H5,H6)}$ = 7 Hz, $^3J_{(H4,H5)}$ = 7 Hz, 1H, H-5$_{Py}$), 5.51 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar-H), 5.50 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar-H), 5.31 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar−H), 5.30 (d, $^3J_{(H,H)}$ = 6 Hz, 1H, Ar-H), 5.11 (d, $^2J_{gem(H,H)}$ = 17 Hz, 1H, NCH_2CO), 4.27 (m, 3H, NCH_2CO, OCH_2CH$_3$), 2.93 (m, 1H, CH(CH$_3$)$_2$), 2.31 (s, 3H, Ar-CH_3), 1.33 (m, 9H, CH(CH_3)$_2$, OCH$_2$CH_3) ppm. ^{13}C{^{1}H} NMR (125.75 MHz, $CDCl_3$, 25 °C): δ 167.3 (C-2), 166.1 (C-3), 160.9 (*C*OOEt), 121.4 (C-4), 117.6 (C-6),

112.7 (C-5), 99.7 (C-Ar), 94.4 (C-Ar), 81.4 (CH-Ar), 80.5 (CH-Ar), 79.3 (CH-Ar), 77.8 (CH-Ar), 61.9 (OCH_2CH_3), 51.3 (NCH_2CO), 31.2 ($CH(CH_3)_2$), 22.5 ($CH(CH_3)_2$), 22.4 ($CH(CH_3)_2$), 18.5 ($Ar-CH_3$), 14.4 (OCH_2CH_3) ppm.

[(η^6-*p*-cymene){*N*-[(ethoxycarbonyl)methyl]-3-oxo-κO-2-(1*H*)-pyridonato-κO}(9-ethylguanine-$\kappa N7$)ruthenium(II)] trifluoromethanesulfonate (5a). A solution of $Ag(CF_3SO_3)$ (20 mg, 0.077 mmol) in H_2O (5 mL) was added to a solution of **1a** (33 mg, 0.070 mmol) in H_2O (15 mL) and stirred for 2 h at RT under light protection. The solution was filtered to remove AgCl, and 9-ethylguanine (14 mg, 0.077 mmol; 9-EtG) was added to the filtrate and stirred for 24 h at RT. The solvent was removed under reduced pressure, and the product was recrystallized from methanol and diethyl ether to give crystals suitable for X-ray diffraction analysis. Yield: 36 mg (67%), mp > 200 °C dec. Elemental analysis: found C, 42.68; H, 4.12; N, 10.94. Calcd for $C_{27}H_{33}N_6O_8F_3SRu$: C, 42.69; H, 4.38; N, 11.06; MS (ESI$^+$) m/z 432.0 $[M - 9EtG]^+$ (100%), 610.8 $[M]^+$ (9%). ^{1}H NMR (500.10 MHz, DMSO-d_4, 25 °C): δ 10.77 (s, 1H, *H*-N1 of 9-EtG), 7.70 (s, 1H, *H*-8 of 9-EtG), 7.03 (dd, $^3J_{(H5,H6)} = 8$ Hz, $^4J_{(H4,H6)} = 1$ Hz, 1H, H-6_{Py}), 6.79 (dd, $^3J_{(H4,H5)} = 7$ Hz, $^4J_{(H4,H6)} = 1$ Hz, 1H, H-4_{Py}), 6.65 (dd, $^3J_{(H5,H6)} = 7$ Hz, $^3J_{(H4,H5)} = 7$ Hz, 1H, H-5_{Py}), 6.41 (brs, 2H, H_2N of 9-EtG), 5.85 (d, $^3J_{(H,H)} = 6$ Hz, 1H, Ar-H), 5.77 (d, $^3J_{(H,H)} = 6$ Hz, 1H, Ar-H), 5.63 (d, $^3J_{(H,H)} = 6$ Hz, 1H, Ar-H), 5.58 (d, $^3J_{(H,H)} = 6$ Hz, 1H, Ar-H), 5.17 (d, $^2J_{gem(H,H)} = 17$ Hz, 1H, NCH_2CO), 5.04 (d, $^2J_{gem(H,H)} = 17$ Hz, 1H, NCH_2-CO), 4.20 (m, 2H, OCH_2CH_3), 3.96 (m, 2H, NCH_2CH_3), 2.84 (m, 1H, $CH(CH_3)_2$), 2.26 (s, 3H, Ar-CH_3), 1.33 (m, 3H, NCH_2-CH_3)1.24 (m, 9H, $CH(CH_3)_2$, OCH_2CH_3) ppm. $^{13}C\{^1H\}$ NMR (125.75 MHz, DMSO-d_4, 25 °C): δ 167.9 (C-2), 165.5 (C-3), 160.0 (COO), 157.3 (C6 of 9-EtG), 154.6 (C2 of 9-EtG), 151.4 (C4 of 9-EtG), 137.4 (C6 of 9-EtG), 129.3 (C-4), 123.5 (C-6), 117.8 (C-5), 117.2 (C5 of 9-EtG), 99.8 (C-Ar), 96.7 (C−Ar), 82.1 (CH-Ar), 81.8 (CH-Ar), 80.4 (CH-Ar), 79.6 (CH-Ar), 62.1 (OCH_2CH_3), 61.7 (NCH_2CH_3), 52.1 (NCH_2CO), 30.99 ($CH(CH_3)_2$), 22.5 (CH-$(CH_3)_2$), 22.4 ($CH(CH_3)_2$), 17.9 (Ar-CH_3), 15.8 (NCH_2CH_3), 14.6 (OCH_2CH_3) ppm.

Hydrolysis Experiments. For hydrolysis studies, compounds **1a**, **1b**, and **4a** (1−2 mg/mL) were dissolved in MeOD-d_4/D_2O (5/95) solution, and the samples were analyzed by ^{1}H NMR spectroscopy. In order to force aquation of the complex, the chlorido ligands were abstracted by addition of 2 equiv of $AgNO_3$ in D_2O, and the solution was filtered to remove AgCl. In both cases, ^{1}H NMR spectra were recorded after 0.5, 24, 48, 72, and 120 h. To study a potential suppression of hydrolysis by the presence of chloride, we dissolved **1a** in 100 mM NaCl solution in D_2O (1−2 mg/mL), and a solution of **1a** in D_2O was placed in a capillary in the same NMR tube for reference.

pK_a Determination. pK_a values were determined by dissolving complexes **1a**, **1b**, and **4a** in MeOD-d_4/D_2O (5/95) solution. The pH values were measured directly in the NMR tubes with a pH meter Eco Scan pH6 equipped with a glass microcombination pH electrode (Orion 9826BN) and calibrated with standard buffer solutions of pH 4.00, 7.00, and 10.00. The pH titration was performed with NaOD (0.4−0.0004% in D_2O) and DNO_3 (0.4−0.0004% in D_2O). The pH values were plotted against the chemical shifts of the Ar_{cym}-H2/H6 protons of the arene ring in the ^{1}H NMR spectra, and the resulting curves were fitted using the Henderson−Hasselbalch equation with Microsoft Office Excel 2003, SP3 (Microsoft Corporation). The experimentally obtained pK_{a*} values were corrected with eq 1, in order to convert the pK_{a*} in D_2O to the corresponding pK_a values in aqueous solutions.[68]

$$pK_a = 0.929\, pK_{a*} + 0.42 \quad (1)$$

Reaction with Biomolecules Monitored by NMR Spectroscopy. 5′-GMP binding experiments were carried out by titrating solutions of the complexes **1a**, **1b**, and **4a** (1−2 mg/mL) in MeOD-d_4/D_2O (5/95) with a 5′-GMP solution (10 mg/mL D_2O) in 50 μL increments. The reaction was monitored by ^{1}H and $^{31}P\{^1H\}$ NMR spectroscopy until unreacted 5′-GMP was detected.

In order to investigate the reactivity of complexes with amino acids, a solution of **1a** (1 mg/mL) in MeOD-d_4/D_2O (5/95) was treated with equimolar amounts of Gly, Ala, Cys, Met, and His (pH of the solutions was in the range 5.5−7.1), and the ^{1}H NMR spectra were recorded after 5 min and 24 h.

Mass Spectrometry Studies on the Binding to Amino Acids and Proteins. Stock solutions of **1a**, **4a**, Gly, Cys, Met, His, ubiquitin (Ub), and cytochrome c (Cyt) (each 400 μM) were prepared in MeOH/H_2O = 10/90. Solutions of **1a** and **4a** were incubated with the selected amino acid at a 1:1 molar ratio for 1, 19, 24, and 48 h at 37 °C. In order to follow the competitive reaction of **1a** or **4a** with amino acids, reaction mixtures with compound:Gly:Met:His:Cys at a molar ratio of 1:1:1:1:1 were prepared and incubated for 19 h. For the protein binding studies, **1a** or **4a** and Ub or Cyt were mixed at 2:1 or 3:1 molar ratios, and aliquots were taken after 1, 19, and 48 h.

The samples were analyzed at concentrations ranging from 10−30 μM. Therefore, they were diluted with MeOH in case of the amino acid incubations and with MeOH/water/formic acid = 50/50/0.1 for the analysis of the protein samples.

ESI mass spectra were recorded on a Bruker esquire$_{3000}$ ion trap mass spectrometer by direct infusion with a flow rate of 4 μL/min. The experimental conditions were as follows: Capillary 4.5 kV, end plate offset 0.5 kV, skimmer 1 45.5 V, skimmer 2 6 V, cap exit offset 78.4 V, and dry temperature 200 °C. The spectra were recorded and processed using ESI Compass 1.3 and DataAnalysis 4.0 software (both Bruker, Bremen, Germany). Deconvolution was obtained by maximum entropy calculations with a mass step of m/z 0.1 and an instrument peak width of 1.

CDK2/Cyclin A Protein Kinase Inhibition Assay. The CDK2 peptide substrate, HHASPRK, and CDK2/Cyclin A protein complex were purchased from Enzo Life Sciences. Adenosine 5′-[γ-ferrocene] triphosphate (Fc-ATP) was synthesized according to the procedure published elsewhere.[69] Gold rod electrodes (99.99% purity) with surface area of 0.02 cm^2 were obtained from CH Instruments. All experiments were conducted in aqueous conditions using ultrapure water (18.2 MΩ cm) from a Millipore Milli-Q system.

Fabrication of Kinase Biosensor. The gold electrodes were cleaned by polishing with slurry of 1 μm Al_2O_3 until a mirror finish was obtained. After 5 min sonication in Milli-Q water, the gold electrodes were rinsed with water and ethanol. The electrodes were then cleaned electrochemically by cyclic voltammetry (CV) in 0.5 M H_2SO_4 in the range 0−1.2 V and then by cycling in the negative potential range from −0.6 to −2.3 V. Next, the gold electrodes were incubated with 2 mM lipoic acid NHS ester solution in ethanol for 3 days at 273 K. After extensive washing with freshly distilled ethanol, the gold electrodes were incubated with a 0.1 mM peptide solution in Milli-Q water for 18 h at 273 K. Consequently, the modified electrodes were rinsed with Milli-Q water and then incubated with 100 mM ethanolamine solution in absolute ethanol for 1 h. Finally, the electrodes were immersed in 10 mM dodecanethiol solution in ethanol for 20 min to block any unmodified gold surface.

Kinase-Catalyzed Phosphorylation Reaction. The peptide-modified gold electrodes were immersed in the kinase assay buffer based on 60 mM HEPES (pH 7.5), 3 mM $MnCl_2$, 3 mM $MgCl_2$, 0.5 μg/μL of PEG 20000, 3 μM sodium ortho-vanadate, 1 μg/mL CDK2/Cylin A protein kinase, and 200 μM Fc-ATP. The phosphorylation reaction was performed for 6 h at 37 °C in a heating block (VWR Scientific, USA). The modified gold

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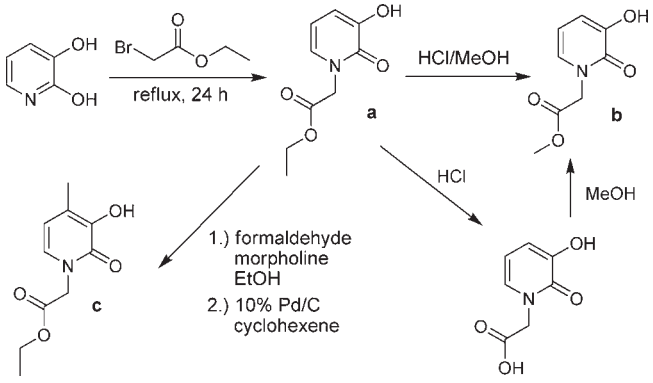
electrodes were washed five times using the kinase assay buffer and 0.1 M phosphate buffer (pH 7.4) prior to the electrochemical measurement. For the inhibitor studies, the CDK2/Cyclin A protein and compounds **1a**, **1b**, or **4a** (1 mM, DMSO) were added to the kinase buffer, while maintaining the working concentration of the protein and inhibitor at 1 μg/mL and 20 μM, respectively. The DMSO concentration was maintained at a low level (< 2%). After 30 min, the kinase reaction was initiated by the addition of Fc-ATP and was followed by the procedure outlined above.

Electrochemical Experiments. All electrochemical experiments were carried out using a CH Instrument 660B system potentiostat (Austin, TX) at a 100 mV/s scan rate unless otherwise specified. The electrochemical measurements were performed in 0.1 M phosphate buffer (pH 7.4). Typical electrochemical experimental set up included a three-electrode system: a modified gold electrode as the working electrode, Ag/AgCl in 3 M KCl as the reference electrode, which was connected with the electrolyte via a salt bridge, and platinum wire as the counter electrode. For each electrode, CV was performed at a scan rate of 100 mV/s, and square-wave voltammetry (SWV) was recorded from 0.2 to 0.6 V, with an amplitude of 25 mV at 20 Hz frequency.

Cytotoxicity in Cancer Cell Lines. Cell Lines and Culture Conditions. CH1 cells (adenocarcinoma of the ovary) were a generous gift from Lloyd R. Kelland, CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, U.K. SW480 (adenocarcinoma of the colon, human) and A549 (nonsmall 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 fetal 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 trypsinization and seeded in 100 μL aliquots MEM supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 4 mM L-glutamine, and 1% nonessential 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 in DMSO, serially diluted in the same medium such that the maximum DMSO content did not exceed 1%, and added in 100 μL aliquots to the microcultures (only the maximum concentration tested was added in 200 μL aliquots after removal of the medium). Cells were then exposed to the test compounds for 96 h. At the end of exposure, all media were replaced by 100 μL/well RPMI1640 culture medium (supplemented with 10% heat-inactivated fetal 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 viable 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 three independent experiments, each comprising three replicates per concentration level.

Scheme 1. Synthetic Strategy to Ligands **a**, **b**, and **c**[60−62,71]



Results and Discussion

Synthesis and Characterization. Hydroxypyridones and their derivatives are a versatile class of ligands and behave as *O*,*O*-bidentates to form 5-membered chelate rings with metals. These 6-membered *N*-heterocycles are readily ionizable by deprotonation of the hydroxyl group, and thereby, the zwitterionic "aromatic" character of the molecule is introduced. They form stable metal complexes with a wide range of bi- or trivalent metals, including Al, Fe, Ga, V, Mo, Zn, etc.[70] In addition to those metals, complexes of 3-hydroxy-4-pyr(id)ones of Ru have been demonstrated to exhibit antitumor activity.[23−29] However, no data for 3-hydroxy-2-pyrones have been reported, a gap which we aim to fill with the present study by synthesizing a series of ruthenium(II)- and osmium(II)-arene complexes of alkoxycarbonylmethyl-3-hydroxy-2-pyridones.

The bidentate ligand 1-[(ethoxycarbonyl)methyl]-3-hydroxy-2(1*H*)-pyridone **a** (Scheme 1) was synthesized straightforwardly by *N*-alkylation of 2,3-dihydroxypyridine with ethyl bromoacetate, acting also as solvent in this reaction.[60] Recrystallization from ethanol (96%) gave rise to the pure compound (about 80% yield). In addition, the simple *N*-alkylation of 2,3-dihydroxypyridine with alkyl halides has been reported, but the synthetic procedures require harsh conditions and often result in poor yields.[60] The methyl ester **b** was obtained in excellent yield (99%) in a one-step procedure via acid-catalyzed transesterification of 1-[(ethoxycarbonyl)-methyl]-3-hydroxy-2-pyridone **a**.[61] Alternatively, **b** is also accessible in two steps, involving in the first step the hydrolysis of ethyl ester **a,** followed by esterification of the pyridone carboxylic acid.[71] In order to introduce regioselectively a methyl substituent at position 4 of the hydroxypyridone scaffold, the Mannich reaction was employed for mono aminomethylation and in a second step, the Mannich base was further transformed to the methyl-substituted *N*-[(ethoxycarbonyl)methyl]-3-hydroxy-4-methyl-2-(1*H*)-pyridone **c** under palladium-catalyzed transfer hydrogenolysis conditions.[62]

Ligands **a**, **b**, and **c** were converted in methanol into the corresponding complexes by activating the alkoxycarbonylmethyl-3-hydroxy-2-pyridones with sodium methoxide. The respective dimers $[(\eta^6\text{-}p\text{-cymene})MX(\mu\text{-}X)]_2$

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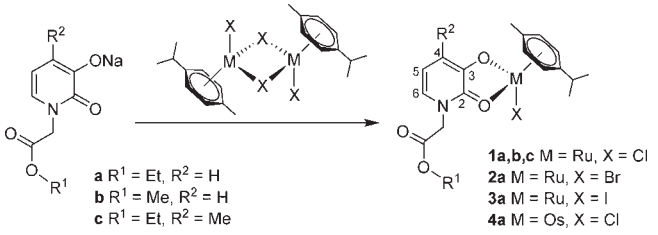
Scheme 2. Synthesis of Complexes from Corresponding Ligands



(X = Cl, Br, I; M = Ru, Os; cym = η^6-*p*-cymene) were added to this solution at RT to yield the products (57−93%) after recrystallization by slow diffusion of diethyl ether into a concentrated solution of dichloromethane (Scheme 2).

The ^{1}H NMR spectra of all complexes in $CDCl_3$ contain doublets assigned to each of the four cym ring protons with a coupling constant of about 6 Hz and two doublets for the cym isopropyl methyl groups due to slow epimerization of the chiral metal center and the presence of nonequivalent aromatic protons. However, in the ^{1}H NMR spectra of **1a** and **1b** in D_2O, conditions in which the complex will hydrolyze quickly to the aqua species (see below), only two doublets for the cym ring protons and a doublet for the two methyl groups of the cym isopropyl are observed.[22] Upon coordination of hydroxypyridone to the ruthenium center, H4, H5, and H6 protons give three sets of signals (two set of doublets in the case of **1c**). In the case of **1c**, the signal attributable to the H4 of hydroxypyridone is no longer present, instead a singlet at δ = 2.19 ppm appears, corresponding to the methyl substituent, accompanied by loss of coupling of H4 to H5 or H6.[62]

For all compounds, the methylene protons of the alkoxycarbonylmethyl-3-hydroxy-2-pyridones become diastereotopic upon complexation and show splitting of their resonances, if analyzed in noncoordinating solvents. The diastereotopic N−CH_2−CO protons adjacent to the pyridone ring give two doublets centered at δ = 5.1 and 4.2 ppm with a geminal coupling constant $^2J_{(H,H)}$ = 17 Hz as expected for an AB system. Similar observations were reported for complexes containing related hydroxypyridones.[46,61,72] If the ^{1}H NMR spectra are recorded in D_2O, the same protons give a singlet at δ = 4.8 ppm, which is comparable to the chemical shift in **a**, **b**, or **c** (δ = about 4.7 ppm).

Crystal structures of **1a** and **1b** were determined by X-ray diffraction analysis. The compounds exhibit pianostool configuration as usually observed for such metal−arene complexes.[13,25,29] The alkoxycarbonylmethyl-3-hydroxy-2-pyridones act as *O*,*O*-chelating bidentate ligands and form a five-membered chelate ring upon binding to the ruthenium(II) center (Figure 1, selected bond lengths and angles are given in Table 2). The ruthenium−centroid$_{arene}$ distances are 1.6397(7) and 1.6427(12) Å, and the Ru—Cl bond lengths are 2.4186(4) and 2.4166(7) Å in **1a** and **1b**, respectively. These parameters are very similar to those observed for a dinuclear RuCl(cym)(3-hydroxy-4-pyridone) complex.[26]

Stability in Aqueous Solution. The aquation of **1a**, **1b**, and **4a** was investigated by ^{1}H NMR spectroscopy in

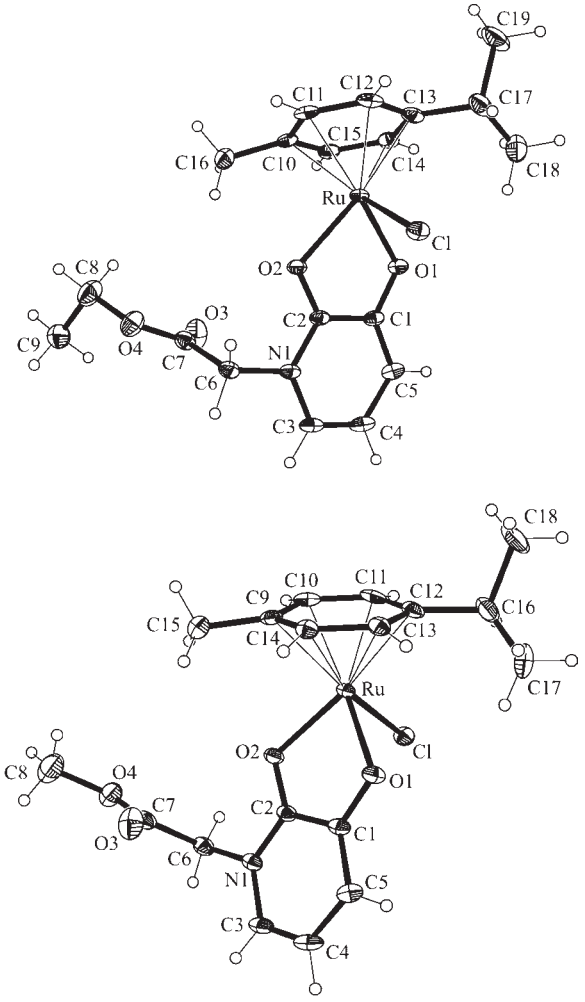


Figure 1. ORTEP plots of **1a** (top) and **1b** (bottom); thermal ellipsoids are drawn at 50% probability level.

D_2O/MeOD-d_4 (95/5) because of limited water solubility. Complexes **1a**, **1b**, and **4a** hydrolyze rapidly upon dissolution in water by exchange of the chlorido ligand with a neutral water molecule and exist predominantly as the charged monoaqua species of the general formula $[M(cym)(HP)(OH_2)]^+$ (see below). This result was confirmed by obtaining similar NMR spectra when hydrolysis was initiated by addition of equimolar amounts of $AgNO_3$. These data suggest immediate aquation after dissolution in aqueous environment. Furthermore, addition of 100 mM NaCl appears not to suppress hydrolysis as shown by ^{1}H NMR spectroscopy. Likewise, fast hydrolysis was also observed for M(arene) complexes containing anionic *O*,*O*-chelating ligands such as acac, maltolato, or other hydroxypyrones in comparison to relative high stability of *O*,*S*-, *O*,*N*-, and *N*,*N*-chelate complexes. This might be due to either the increased electron density at the metal center or possible interactions between hydrogen atoms of coordinated H_2O and the oxygen atoms of the *O*,*O*-chelating ligand, which in the case of acac was also proposed by DFT calculations.[4,24,73] When comparing the hydrolysis behavior of Ru and Os analogues, the Os compound **4a** is less water soluble and hydrolyzes

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Table 2. Selected Bond Lengths (Å) and Bond Angles (deg) for **1a**, **1b**, and **5a**

	1a	1b	5a
Ru−(O=C)	2.0716(12)	2.0686(16)	2.0711(15)
Ru−(O−C)	2.1294(12)	2.1323(17)	2.0941(15)
Ru−Cl[a]	2.4186(4)	2.4166(7)	2.1227(17)
Ru(1)−centroid	1.6397(7)	1.6427(12)	1.6407(10)
O−Ru−O	78.75(5)	78.96(7)	79.82(6)
(C=O)−Ru−Cl[a]	85.51(4)	84.73(5)	82.03(6)
(C−O)−Ru−Cl[a]	84.51(3)	84.08(5)	82.60(6)

[a] In the case of **5a**, the values for the Ru−N bond length and the O−Ru−N angles are given.

significantly slower than the ruthenium analogue **1a** because of the higher lability of Ru than of Os complexes. The aqua species $[M(cym)(HP)(OH_2)]^+$ are stable for more than five days, and in contrast to analogous hydroxypyrone complexes,[22] no dimeric species of $[(cym)_2Ru_2(\mu-OH)_3]^+$ are formed.[23,74] The NMR data were essentially confirmed by electrospray ionization mass spectrometry (ESI-MS). Both **1a** and **4a** exhibit similar behavior in solution, with the chlorido ligand being released within an hour. This fact is supported by the presence of base peaks assignable to $[M(cym)(HP)]^+$ in the MS spectra at m/z 432.3 and 522.3 for **1a** and **4a**, respectively, and the peaks showing the characteristic isotope pattern for these metal compounds. Furthermore, transesterification to the methyl ester and hydrolysis of the ester moiety occurred for **1a** and **4a** in presence of methanol, however at low relative abundance (e.g., for **1a** at m/z 418.3 [10%] and 404.2 [5%]). These side products were observed at increased relative abundance during the protein binding experiments (see below) in the presence of formic acid (Figure S1 of the Supporting Information).

The pK_a values of **1a**, **1b**, and **4a** were determined by titrating the aqua species of the respective complexes with NaOD to afford the corresponding hydroxido complex and monitoring the deprotonation process by ^{1}H NMR spectroscopy (Table 3). The aqua species were generated by dissolving the complexes in D_2O containing 5% MeOD-d_4. The pK_a values were determined by plotting the chemical shifts of the proton signal of the arene ring Ar_{cym}-H2/H6 against the pH value (e.g., from 5.67 ppm at pH 2.78 to 5.29 at pH 10.03 for **1a**), and the inflection points of the sigmoid curves give the pK_{a*} values, which were corrected using eq 1 in order to consider the difference between D_2O and water and to yield the pK_a. The pK_a values determined for **1a**, **1b**, and **4a** are in a similar range as those of the structurally related hydroxypyr(id)ones.[22,25,29] As reported earlier, aqua ligands coordinated to osmium are significantly more acidic than at ruthenium centers.[22,75] Note that pK_a values strongly depend on the first coordination sphere and can easily be modified by replacing *O*,*O*-chelators by bidentate *N*,*N* ligands, which leads to a significant decrease in the pK_a values.[75,76]

Table 3. pK_a Values of **1a**, **1b**, and **4a** and Their in Vitro Anticancer Activity in Human Ovarian Cancer (CH1), Colon Cancer (SW480), and Nonsmall Cell Lung Cancer (A549) Cells (exposure time 96 h)[a]

		IC_{50} values/μM		
compound	pK_a	CH1	SW480	A549
1a	8.97	242 ± 29	> 320	> 320
1b	9.46	232 ± 7	> 320	> 320
4a	7.70	129 ± 7	> 320	> 320

[a] Values are means ± standard deviations obtained from at least three independent experiments.

Amino Acids as Binding Partners for Hydroxypyridone Complexes. Most metallodrugs are administered intravenously, and hence, they encounter a number of reactive biomolecules such as proteins in the bloodstream. Serum proteins can play a divergent role either in delivery of metal-based anticancer drugs to their cellular targets or in deactivating them even before reaching the target(s).[9,77] Therefore, the reaction of **1a** with the amino acids Gly, Ala, His, Met, and Cys was investigated to gain insight into the reactivity to biomolecules and potential metabolization. Histidine and methionine as well as cysteine have been chosen because they are known to undergo favorable binding interactions with ruthenium(II) anticancer complexes, whereas Gly and Ala served as nonselective references. For ^{1}H NMR studies, the amino acids were incubated with **1a** at equimolar ratios in D_2O/MeOH-d_4 (95/5), and the reaction was monitored for 24 h. It appears that the bidentate hydroxypyridone ligand was replaced by the respective amino acid within 24 h as signals assignable to the released hydroxypyridones appeared in the ^{1}H NMR spectra. Notably, Cys decomposes the complex within minutes, probably due to the strong *trans*-effect of the thiol functionality. A similar observation has been reported for related pyrone-derived complexes.[25] Similarly, the reaction of **1a** with 1 equiv of Met and His takes place immediately with quantitative release of the pyridone ligand within 24 h, possibly due to the ability of both amino acids to act as bidentate or tridentate chelating ligands (with the thioether of Met and *N*1 or *N*3 atoms of the imidazole moiety of His as third donors in addition to NH_2 and COOH). This hypothesis is supported by the mass spectrum of the reaction between **1a** and His in which the peak at m/z 346.1, which appeared after 48 h, was assigned to $[Ru(cym)(His - CO_2) - 2H]^+$ (1%). This indicates *N*,*N* chelation of His to the metal center. The reaction of Gly with **1a** was significantly slower than with all the other investigated amino acids. In the ^{1}H NMR spectra recorded after 24 h, there appeared two doublets at δ = 3.11 and 2.94 ppm with a geminal coupling constant of $^2J_{(H,H)}$ = 17 Hz. These signals were assigned to the Gly-CH_2 protons, which become diastereotopic upon coordination of Gly as an *N*,*O*-chelating ligand to the Ru(II)-arene center forming a five-membered ring. In analogy to related complexes,[24,25] no ruthenium dimer signals appeared within 24 h in the presence of amino acids (in ^{1}H NMR spectra signals at approximately 5.0 and 5.5 ppm would be indicative for this process).

In order to investigate the interaction of **1a** with amino acids (aa) by means of ESI-MS, **1a** was incubated with

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one mol equiv of Gly, His, Met, or Cys at 37 °C in water/methanol (90/10). The samples were analyzed after 1, 24, and 48 h, and the trend to replace the pyridone ligand is already observable within1 h, however to different extents and with differing kinetics. After 48 h incubation of **1a** with Met or His, the major products were identified as $[Ru(cym)(aa) - H]^+$ as were for Cys and Gly but at lower relative abundance. To perform a competition experiment, we incubated **1a** with Gly, His, Cys, and Met at a molar ratio of 1:1:1:1:1 for 19 h, and the following trend for the formation of amino acid adducts was observed: Met ≥ His ≫ Cys > Gly (Table S1 and Figure S2 of the Supporting Information). The preference for Met and His might indicate enhanced stability of the formed adducts as compared to their Gly and Cys counterparts. The obvious feature of Cys, Met, and His as compared to Gly is the side chain with soft donors that can be directly involved in coordination to the metal center. Furthermore, when comparing Cys with His and Met, the side chains of the latter two aa are more flexible to act as *N*,*O*,*S* or *N*,*O*,*N* chelating ligands, whereas the Cys complex is more constrained. These observations are also supported by bis-aa adduct formation during the reaction of **1a** with Gly found at *m*/*z* 385.9 $[Ru(cym)(Gly)_2]^+$ and 367.9 $[Ru(cym)(Gly)_2-H_2O]^+$. In addition, the dinuclear species $[(cym)_2Ru_2(Gly) + H_2O]^+$was detected at *m*/*z* 654.9 (5%). However, species containing both Gly and the pyridone ligand were not observed. In analogy to the ^{1}H NMR experiments, Cys appears to degrade **1a**, which might be a reason for the low relative abundance of Cys adducts in the mass spectrum, and several peaks appeared in the range *m*/*z* 500−950, one of which was identified as dimeric $[(cym)_2Ru_2(Cys)_2 + H_2O]^+$ at *m*/*z* 731.8.

The reactivity of the osmium analogue **4a** toward amino acids is similar to that of **1a** as is the result of the competition experiment (Met≈His > Cys ≫ Gly). Again, the pyridone ligand is replaced in each case by the respective amino acid giving rise to $[Os(cym)(aa) - H]^+$ (aa = Met, His, Cys, Gly). Os has an even greater affinity for softer ligands compared to Ru, but ligand exchange rates are lower and replacement of the pyridone ligand is thus slower. Interesting features were observed in the mass spectrum of the reaction mixture containing His. In analogy to **1a**, a signal at *m*/*z* 480.3 $[Os^{II}(cym)(His) - H]^+$ was observed which was partly overlapping with a peak with the most abundant isotope at *m*/*z* 478.3. This signal might be related to $Os^{II/IV}$ oxidation (occurring probably during the ionization process in the ESI-MS source), which requires double deprotonation to balance the charge contributed by the metal center (Figure S3 of the Supporting Information). Furthermore, again a decarboxylated species was observed at *m*/*z* 434.4 $[Os^{IV}(cym)(His - CO_2) - 3H]^+$ (10%), and the peak at *m*/*z* 462.4 was assigned to $[Os^{II}(cym)(His - OH) - 2H]^+$ (10%). In the reaction with Gly, **4a** forms dinuclear species not observed for **1a** such as $[(cym)Os_2(\mu\text{-}OMe)_3]^+$ (*m*/*z* 743.4; 15%) and $[(cym)Os_2(\mu\text{-}OMe)_2(\mu-OH)]^+$ (*m*/*z* 729.3; 22%), probably because Gly is only a weak binding partner.

Reaction of 1a and 4a with Ubiquitin and Cytochrome c. In order to estimate the binding to proteins as potential targets in the bloodstream, we incubated **1a** and **4a** at molar ratios of 2:1 with ubiquitin (Ub) and 3:1 with cytochrome c (Cyt). The reaction mixtures were analyzed with an ESI-ion trap-MS, and the data was deconvoluted with the maximum entropy deconvolution algorithm. Ion traps deliver spectra of relatively low resolution; however, the identity of the formed species can be clearly identified in this case. Such ESI-MS experiments have been successfully employed for a diversity of biomolecule binding studies, in particular of metal complexes to DNA and proteins, including the characterization of the formed adducts and more sophisticated binding site characterization.[29,78−97]

1a reacts quickly with Ub, and already after 1 h, the peak at 8565 ± 2 Da assigned to unreacted ubiquitin has vanished, giving rise to a signal at 8802 ± 2 Da (100%), corresponding to the monoruthenated adduct $[Ub + Ru(cym)]^+$ and a bisadduct $[Ub + 2\ Ru(cym) - H]^+$ at 9035 ± 2 Da (8% after 48 h), again accompanied by loss of the pyridone ligand. Various other signals appeared at masses >9 kDa, stemming most probably from tertiary adducts formed with solvent molecules, which were gone after longer incubation, and as most intense signals remained after 19 and 48 h peaks at *m*/*z* 8802 ± 2 and *m*/*z* 9035 ± 2 Da assigned to $[Ub + Ru(cym)]^+$ (100%) and $[Ub + 2\ Ru(cym) - H]^+$ (7 and 8%), respectively.

4a reacts with ubiquitin in a similar manner as observed for **1a** but at a lower rate. Consequently, **4a** binds to ubiquitin to form [Ub + Os(cym)] adducts (Figure 2). After 19 h reaction time, the ubiquitin parent mass was

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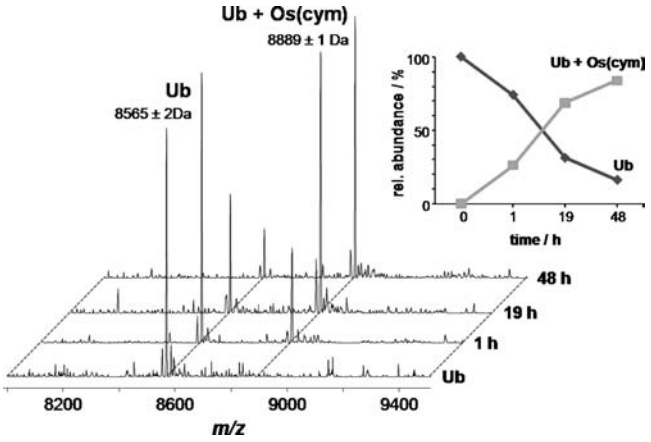


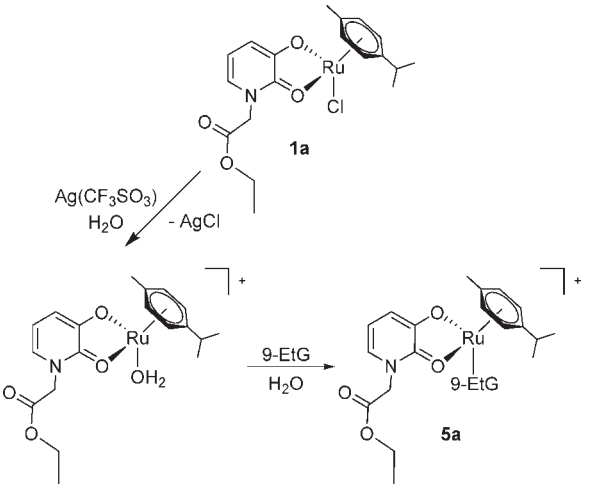
Figure 2. Time course for the reaction of **4a** with Ub. Inset shows the relative changes in the signal intensities for Ub and its Os(cym) adduct.

still present at 40% relative intensity, and in contrast to **1a**, no bisadduct was observable within this time period. Prolonging the reaction time to 48 h showed that the intensity of the unreacted protein decreased to 19% relative to the monoadduct. It appears as the reaction proceeds by forming only a limited number of adducts, whereas in the reaction of Ub with **1a** a multitude of species were detected. This might be related to the higher stability of the Os compound as compared to that of the Ru analogue.

In addition to the studies with Ub with potential His and Met binding sites, the reaction with cytochrome c (Cyt), a Cys containing heme protein, was monitored for both **1a** and **4a** by ESI-MS. Within 1 h incubation, no adducts were observed, but over the time course of the reaction, a series of different species was detected. After 19 h, a monoruthenated Cyt adduct was observed at 12597 ± 1 Da at similar relative intensity as unreacted Cyt (12363 ± 1 Da). Furthermore, a series of higher adducts were detected, including the bisadduct at 12831 ± 1 Da. As was the case for the reaction with ubiquitin, the pyridone ligand of **1a** is cleaved off during the reaction. The reaction of Cyt with **4a** revealed again higher stability of the Os compound. After 1 h incubation, the Cyt peak is the most abundant in the mass spectrum, with a monoadduct visible at 12686 ± 1 Da and possibly a series of aqua species, e.g., a signal at 12732 ± 1 Da was attributed to a trisaqua adduct. It seems as these species are mainly intermediates in the binding process and are converted into the trifunctionally bonded [Cyt + Os(cym)] as can be seen after an incubation time of 19 h, when the most intense signal corresponds already to the monometalated protein at 12686 ± 1 Da (100%) versus Cyt (50%), and no higher adducts were observed.

DNA as a Potential Target. Because DNA is thought to be one of the important targets for metal-based anticancer agents, the reactions of **1a**, **1b**, and **4a** with 5′-GMP were investigated by ^{1}H NMR spectroscopy in D_2O/MeOH (90/10), conditions under which immediately the aqua species are formed (see above). The reaction occurred in a quantitative manner within minutes, and the resulting adducts were stable in solution for more than

Scheme 3. Replacement of the Chlorido Ligand by 9-EtG; Counterion $CF_3SO_3^-$ Has Been Omitted for Clarity



48 h. Upon addition of the metal complex, the H8 signal of 5′-GMP shifts from δ = 8.11 to 7.76 and 7.72 ppm due to the formation of isomers.[98] This change in chemical shift indicates binding to the *N*7 of the guanine moiety and implies that DNA may be a possible target for these Ru(II)−arene complexes as proposed for related organometallic ruthenium(II) compounds.[22]

In order to characterize the interaction of compound **1a** with guanine residues more in detail, **1a** was reacted with 9-ethyl guanine (9-EtG) to yield **5a**. The chlorido ligand of **1a** was abstracted by addition of $Ag(CF_3SO_3)$ (Scheme 3), AgCl was removed by filtration, and 9-ethylguanine was added to the filtrate and stirred for 24 h to afford **5a**. The molecular structure of **5a** was determined by X-ray diffraction analysis of a crystal grown from methanol/diethyl ether by the diffusion technique. This is the first example of a crystal structure of a Ru(arene)[hydroxypyr(id)one] complex bearing a 9-EtG moiety. As implied from the results of the NMR studies, the 9-ethylguanine is coordinated to ruthenium via its *N*7 atom (Figure 3). The Ru−$N7_{Gua}$ bond length in **5a** is 2.1227(17) Å and hence slightly shorter than that observed for a 9-EtG adduct of a Ph_2acac-Ru(cymene) complex [2.140(3) and 2.126(3) Å], whereas the Ru−O bond lengths are rather similar (for key bond lengths and angles see Table 2).[73]

Two molecules of **5a** are linked by hydrogen bonds between *N*3 and NH_2 of neighboring guanines to form a planar structure between the Ru centers, whereas in other cases a noncoplanar arrangement was observed.[99] In **5a**, both HP ligands point on different sides of the plane, whereas in a structure of a complex bearing Ph_2acac, both ligands are on the same side of the plane.[73] Furthermore, *N*1-H and NH_2 of 9-EtG are involved in hydrogen bonding to two oxygen atoms of the $CF_3SO_3^-$ counterion (Figure 3 and Figure S4 of the Supporting Information).

Evaluation of the in Vitro Anticancer Activity and CDK2 Inhibition. The antiproliferative activity of **1a**, **1b**, and **4a** was determined in human SW480 colon adenocarcinoma, CH1 ovarian cancer, and A549 nonsmall cell lung cancer cells by means of the MTT assay (Table 3); **1c**, **2a**, and **3a**

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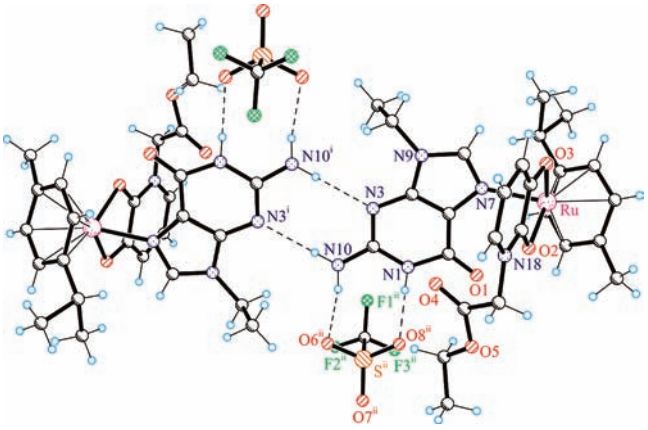

Figure 3. Part of the crystal structure of **5a** showing the coordinated 9-EtG pairing and short contacts to $CF_3SO_3^-$ counteranion via intermolecular hydrogen-bonding interactions N10−H···N3^i [N10···N3^i 3.032 Å, N10−H···N3^i 178.2°], N1−H···O8^{ii} [N1···O8^{ii} 2.909 Å, N1−H···O8^{ii} 177.2°], and N10−H···O6^{ii} [N10···O6^{ii} 3.006 Å, N10−H···O6^{ii} 169.1°]. Atoms marked with i and ii are at symmetry positions $-x, -y + 2, -z + 1$, and $-x + 1, -y + 1, -z + 1$.

were not sufficiently soluble in aqueous dilutions of DMSO stocks to a maximum DMSO content of 1% to be included in the biological assays. The ruthenium complexes **1a** and **1b** showed low anticancer activity in the most sensitive cell line with IC_{50} values of 242 and 232 μM, respectively, while that of the osmium complex **4a** is nearly 2 times lower (129 μM). This is one of the relatively few known examples that an osmium complex is more cytotoxic than its ruthenium analogue.

In an attempt to determine a possible CDK2/Cyclin A kinase inhibition of the Ru and Os complexes, a substrate peptide (HHASPRK) was immobilized onto an Au surface and Fc-ATP was employed as a cosubstrate in the kinase phosphorylation reaction assay.[69,100] Following the kinase-catalyzed phosphorylation reaction on the surface in the presence of the Fc-ATP bioconjugate, the electrochemical response was measured by cyclic voltammetry (CV) and square-wave voltammetry (SWV). The organometallic species do not exhibit redox activity in the region of interest, which makes the electrochemical peptide biosensor a valuable probe for inhibitor screening. The Ru and Os complexes inhibit the kinase activity at a concentration of 20 μM to a comparable extent as the known inhibitor R-roscovitine (Figures S6−S8 of the Supporting Information), which is currently undergoing clinical trials.[101] These data might indicate an interesting mode of action for this type of complexes, though the in vitro anticancer activity is only moderate. However, in vitro anticancer potency appears not to be a prerequisite in particular for ruthenium drug candidates, considering that NAMI-A failed the National Cancer Institute screening but entered clinical trials, and RAPTA compounds are excellent inhibitors of metastasis in vivo.[102−105] These facts support the requirement of improved biological tools with higher predictability in order to establish correlations between in vitro anticancer activity, target modulation, and efficacy in humans.

Conclusions

Organometallic compounds have emerged to an extensively studied class of anticancer chemotherapy drug candidates. Of particular interest are Ru(arene) and Os(arene) compounds, and on the basis of these scaffolds a series of compounds with a diversity of modes of action has been designed. Herein, we present Ru and Os complexes with hydroxypyridones, a compound family that can act as versatile ligands because of many options for derivatization, leading to compounds with particular chemical reactivity and biological properties. A series of Ru− and Os−(η^6-*p*-cymene) compounds was synthesized and characterized by standard methods. NMR studies revealed that the compounds hydrolyze quickly to the respective aqua species with pK_a values in the range 7.7−9.5 and the Os compound at the lower end of this scale. Those aqua species are stable for several days but react quickly, however to a different extent with the amino acids Met, Cys, His, Gly, and Ala, and form M(η^6-*p*-cymene)(amino acid) adducts upon loss of the hydroxypyridone ligand. The same types of adducts were observed in the reactions with ubiquitin and cytochrome c as determined by electrospray ionization mass spectrometry. In general, the Os complex **4a** appeared more stable than its Ru counterpart **1a**, indicated by a lower number of different adducts formed initially after incubation with the proteins. Longer reaction times lead in both cases to the formation of defined mono- or even bisadducts of the type [protein + M(η^6-*p*-cymene)]. In contrast to amino acids and proteins, 5′-GMP and 9-ethylguanine, employed as DNA models, did not induce cleavage of the metal−hydroxypyridone bond but coordinate monodentately via their *N*7 atoms to the metal center. In vitro anticancer activity assays in a series of human tumor cell lines revealed only low potency to inhibit the growth of tumor cells but potent CDK2/Cyclin A protein kinase inhibition by the Ru and Os complexes (similar to the drug candidate roscovitine). It appears as in this particular case (compare introduction) the reactivity of the compounds to biomolecules determines their in vitro anticancer activity, though a broader panel of complexes with different substitution pattern is required to draw definite conclusions.

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Supporting Information Available: Amino acid and protein mass spectral data, molecular structure of **5a**, and CDK inhibition data as well as concentration−effect curves. This material is available free of charge via the Internet at http://pubs.acs.org.

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