Differential impact on oral cancer cell viability for three carboxylate-stabilized rhenium(I) tricarbonyl centers supported by 2,2’-bipyridine: one-pot synthesis, a structure, and proton-catalyzed carboxylate ligand substitution

Lyndsey Buren[1], Emily Tumbaco[1], Alyssa Piesco[1], Justin Irvin[1], Thomas J. Emge[2,], Jeffrey H. Weisburg[3], Gregory A. Moehring[1,*], and Datta V. Naik[1]

[1]Department of Chemistry and Physics, Monmouth University, West Long Branch, NJ 07764, USA

[2]Department of Chemistry and Chemical Biology, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA

[3]Department of Biology, Monmouth University, West Long Branch, NJ 07764, USA

*author to whom correspondence should be addressed

*Gregory A. Moehring: gmoehrin@monmouth.edu

ABSTRACT

A set of six carboxylate-stabilized rhenium(I) tricarbonyl complexes supported by a 2,2'-bipyridine (bpy) ligand, $Re(O_2CR)(CO)_3(bpy)$ (R = H, CH_3, CHF_2, *R*- or *S*-$CHBrCH(CH_3)_2$, and C_5H_{11}), were prepared by acidolysis of the complex $Re(OCO_2C_5H_{11})(CO)_3(bpy)$ with the appropriate carboxylic acid and characterized by ^1H and ^{13}C-$\{^1H\}$ NMR and IR spectroscopy. The crystal structure of the complex, Re[*R*-$O_2CCHBrCH(CH_3)2$]$(CO)_3(bpy)$, was determined by X-ray crystallography. An alternate one-pot route to the carboxylate-stabilized rhenium(I) complexes $Re(O_2CR')(CO)_3(bpy)$ (R' = CH_3 or C_6H_5) and $Re(O_2CCH_3)(CO)_3$(1,10-pheanthroline), which starts with $Re_2(CO)_{10}$ and in which an ester solvent serves as the source of the carboxylate ligand, was also developed. Cell viability tests on three carboxylate-stabilized rhenium(I) complexes (R = H, CH_3, or CHF_2), using the HSC-2 oral cancer cell line, found different levels of cytotoxicity for each complex. NMR studies of the carboxylate ligand substitution reaction found that the reaction is catalyzed by protons. In a chloride-rich NMR solution, substitution of the carboxylate ligand leads to either a chloride-stabilized neutral complex (major product) or to a water-stabilized cation (minor product). Cytotoxicity results correlate positively with the K_b value of the carboxylate ligand. Apparently, the more substitutionally inert the carboxylate-stabilized complex is in a chloride-rich environment (similar to extracellular fluid) the greater the amount of cytotoxic $[Re(CO)_3(bpy)(H_2O)]^+$ that forms in the cytosol.

Keywords:

Rhenium, Cytotoxicity, HSC-2 oral cancer cells, Crystal structure, Carboxylate ligand substitution, One-pot synthesis

1. Introduction

Rhenium(I) complexes which include three carbonyl ligands and a diimine ligand continue to draw interest as both cell imaging agents [1-10] and for their cytotoxicity [5,9-21]. Many such reports also examine the mechanism of the exhibited cytotoxicity [2,3,6,9,12,17,18,20-22]. A subset of rhenium(I) complexes supported by diimine ligands include a monodentate carboxylate ligand in their coordination sphere [6,15,18,23]. A convenient synthetic route to such complexes where the sixth coordination site at rhenium is occupied by a carboxylate ligand has been reported (Equations 1 and 2) [23,24]. Reportedly such complexes lose their carboxylate ligand in solution and form a water-substituted cation that can serve as an active cytotoxin (Equation 3) [6]. The mode of action for the water-stabilized rhenium(I) cytotoxin may involve the binding of rhenium to a nitrogen center within the cell [9].

Equation 1:

$$Re_2(CO)_{10} + 2\ bpy + 2\ CO_2 + 2\ C_5H_{11}OH \rightarrow 2\ Re(OCO_2C_5H_{11})(CO)_3(bpy) + 4\ CO + H_2$$

Equation 2:

$$Re(O_2COC_5H_{11})(CO)_3(diimine) + RCO_2H \rightarrow Re(O_2CR)(CO)_3(diimine) + CO_2 + C_5H_{11}OH$$

Equation 3:

$$Re(O_2CR)(CO)_3(bpy) + H_2O \rightarrow [Re(CO)_3(bpy)(H_2O)]^+ + (RCO_2)^-$$

The carboxylate-stabilized rhenium(I) complexes in the above reports focus primarily on complexes that may interact with cells in two different fashions. Dichloroacetate (DCA) was used in one study [6] while nonsteroidal anti-inflammatory drugs (NSAIDs) were common ligands for rhenium in the other three studies [15,18,23]. Either DCA or NSAIDs can produce an antiproliferative effect on cancer cells. Rhenium complexes in which the carboxylate ligand has been substituted can also have an antiproliferative effect on cancer cells. The study which included DCA found the largest antiproliferative effect for a complex in which DCA was bound to the rhenium-containing complex by an amide linkage to a rhenium-bound pyridine ligand rather than when DCA was bound directly to rhenium [6]. Two of the reports which examined rhenium-bound NSAIDs included DNA-binding studies which indicated partial intercalation of the rhenium complex with DNA at the minor groove [15,23]. The remaining study found that the carboxylate-stabilized rhenium(I) complex caused an increase in reactive oxygen species at the mitochondria [18]. Additionally, one study which examined a variety of rhenium-bound NSAIDs found that the cytotoxicity of the complexes increased as the lipophilicity of the complex increased [23].

The effect of lipophilicity on cytotoxicity suggested that an examination of carboxylate ligand variation with respect to cytotoxicity might be productive. Differences in cytotoxicity associated with lipophilicity due to carboxylate ligands could indicate that carboxylate ligand identity affects cytotoxicity in other fashions as well. The goal of this work was to examine other potential impacts of carboxylate ligand identity on the cytotoxicity of carboxylate-stabilized rhenium-complexes. Towards that goal we looked to vary carboxylate alkyl chain length, examine an enantiomeric pair of complexes, vary the value of K_b for the carboxylate ligands, and include at least one aromatic-containing carboxylate ligand in this study. Human oral cancer cells (HSC-2) and normal human oral cells (HF-1) were used to examine the cytotoxicity of the carboxylate-stabilized rhenium(I) complexes.

2. Experimental

2.1. Materials and methods.

The complex $Re(O_2COC_5H_{11})(CO)_3(bpy)$ (Complex 7) was prepared according to a standard literature preparation [16]. Other reagents were used as received from the vendor. Human squamous carcinoma HSC-2 cells derived from the floor of the oral cavity were provided by Dr. Hiroshi Sakagami of Meikai University School of Dentistry. Human normal gingival HF-1 fibroblasts used in this study were provided by Dr. Peter Sacks of New York University School of Dentistry. Growth medium for cells consisted of Dulbecco's modified Eagle's medium (DMEM) with pyruvate, supplemented with 10% fetal bovine serum, 50 U/ml penicillin G, and 50 U/ml streptomycin sulfate. Cultures were maintained in a humidified atmosphere with 5.0% CO_2 at 37˚C. Cell dissociation was achieved with 0.1% trypsin-0.04% EDTA. To ensure that the cell lines used in the experiments were not infected with *Mycoplasma, Acholeplasma*, and *Ureaplasma* species, a test using Lookout Mycoplasma PCR Detection Kit was performed and no contamination was observed (Supporting Material). The protocol included with the kit was followed. Further experimental details are available in the Supporting Material.

2.2. Cell Viability (WST-1) Assay

As per the kit protocol, cell viability was determined by the stability of the cells to metabolically reduce the tetrazolium salt to a dark red formazan dye using a WST-1 kit (Millipore Sigma). Individual wells of a 96-well tissue culture plate were seeded with 1×10^4 cells in 0.1 mL of growth media and incubated overnight at 37 ºC and 5 % CO_2 to allow the cells to adhere. The growth media was removed and replaced with DMEM with or without various concentration of rhenium compound. After 24 hours of exposure at 37 ºC, WST-1 was added to the cells and allowed to incubate for a maximum of 2 hours at 37 ºC. Viability was determined by relative absorbance at 440 nm on a BioTek Cytation 5 microtiter plate spectrophotometer.

2.3. Acidolysis preparations of complexes.

2.3.1. $Re(O_2CH)(CO)_3(bpy)$ (Complex 1).

Complex 1 was prepared by dissolving 0.050 g (0.090 mmol) of Complex 7 in 5 mL of dichloromethane. The resultant solution was treated with 5.0 µL (0.13 mmol) of formic acid and stirred for 15 min. Complex 1 was precipitated from the solution by the addition of 10 mL of methyl, *t*-butyl ether (MTBE) and 25 mL of pentane. The yellow powder was filtered and dried under vacuum (0.029 g, 0.049 mmol, 54%). Anal. Calcd for $C_{14}H_9N_2O_5Re$: C, 35.67; H, 1.92; N, 5.94. Found: C, 35.46; H 1.94; N, 6.08. ^1H NMR (d_6-DMSO): 9.05 (m, 2H, bpy), 8.77 (m, 2H, bpy) 8.36 (td 7.7 and 1.5 Hz, 2H, bpy) 7.77 (m, 2H, bpy), 7.763 (s, 1H, formate). ^{13}C-{^1H} NMR (d_6-DMSO): 198.62 (s, CO), 195.20 (S, CO), 166.49 (s, formate), 155.67 (s, bpy), 153.84 (s, bpy), 141.05 (s, bpy). 128.29 (s, bpy), 124.70 (s, bpy). IR (ATR cm^{-1}): 3082 (w), 2808 (w), 2714 (w), 2014 (vs), 1912 (s), 1864 (vs), 1630 (s), 1601 (m), 1494 (w), 1473 (m), 1445 (m), 1380(w), 1291 (m), 1279 (m), 1176 (vw), 1159 (w), 1105 (vw), 1074 (w), 1048 (vw), 1034 (vw), 905 (w), 808 (vw), 773 (s), 733 (m), 663 (vw), 648 (m), 626 (m), 534 (m), 486 (w), 446 (vw), 422(m).

2.3.2. Re(O_2CCH_3)(CO)$_3$(bpy) (Complex 2).

Complex 2 was prepared in a method similar to complex 1 using 0.100 g (0.179 mmol) of Complex 7 mixed with 0.50 mL (8.7 mmol) of glacial acetic acid in 5 mL of tetrahydrofuran and a mixing time of 30 min. Complex 3 was precipitated by adding 10 mL of MTBE and 10 mL of pentane to the solution. The precipitate of Complex 2 (0.048 g, 0.10 mmol, 57%) was collected by filtration and washed with two 5 mL portions of MTBE. Anal. Calcd for $C_{15}H_{11}N_2O_5Re$: C, 37.11; H, 2.28; N, 5.77. Found: C, 36.92; H 2.27; N, 5.85. ^1H NMR (d_6-DMSO): 9.04 (m, 2H, bpy), 8.74 (d 8.2 Hz, 2H, bpy), 8.34 (td 7.8 and 1.4 Hz, 2H, bpy), 7.76 (m, 2H, bpy), 1.39 (s, 3H, acetate). ^{13}C-{^1H} NMR (d_6-DMSO): 198.99 (s, CO), 194.97 (s, CO), 174.60 (s, -OC(O)-), 155.61 (s, bpy), 153.88 (s, bpy), 140.86 (s, bpy), 128.15 (s, bpy), 124.54 (s, bpy), 23.59 (s, CH3). IR (ATR cm^{-1}). 3113 (vw), 3087 (vw), 2015 (s), 1885 (vs), 1619 (m), 1601 (m), 1494 (vw), 1473 (m), 1444 (m), 1367 (s), 1316 (s), 1245 (w), 1162 (w), 1122 (vw), 1106 (vw), 1077 (vw), 1033 (w), 1017 (w), 969 (vw), 910 (vw), 805 (vw), 771 (s), 732 (m), 675 (m), 647 (m), 628 (m), 538 (m), 511 (vw), 486 (m), 463 (w), 444 (vw), 420 (m).

2.3.3. Re(O_2CCHF_2)(CO)$_3$(bpy) (Complex 3).

Complex 3 was prepared by dissolving 0.110 g (0.197 mmol) of Complex 7 in 5 mL of tetrahydrofuran. A 0.50 mL (7.9 mmol) portion of difluoroacetic acid was added to the solution which was then allowed to stir for 30 min. After stirring, a 5 mL portion of n-pentane was added to the solution and a precipitate of Complex 3 formed. The precipitate, (0.077 g, 0.15 mmol, 76%) was filtered and washed with 5 mL portions of diethyl ether. Anal. Calcd for $C_{15}H_9F_2N_2O_5Re$: C, 34.55; H, 1.74; N, 5.37. Found: C, 34.72; H 1.84; N, 5.31. ^1H NMR (d_6-DMSO): 9.07 (m, 2H, bpy), 8.76 (d 8.2 Hz, 2H, bpy), 8.37 (t 7.8 Hz, 2H, bpy), 7.78 (m, 2H, bpy), 5.59 (t $^2J_{HF}$ = 54.7 Hz, 1H, CHF_2). ^{13}C-{^1H} NMR (d_6-DMSO): 198.11 (s, CO), 194.44 (s, CO), 166.69 (t $^2J_{CF}$ = 51.2 Hz, -OC(O)-), 155.71 (s, bpy), 154.01 (s, bpy), 141.24 (s, bpy), 128.35 (s, bpy), 124.65 (s, bpy), 108.33 (t $^1J_{CF}$ = 245.0 Hz, CHF_2). IR (ATR cm^{-1}): 3091 (vw), 2017 (vs), 1881 (vs), 1666 (s), 1604 (m), 1496 (vw), 1474 (m), 1447 (m), 1409 (m), 1316 (s), 1248 (w), 1164 (w), 1101 (m), 1071 (m), 1034 (m), 971 (vw), 946 (w), 808 (m), 772 (s), 732 (m), 662 (w), 648 (m), 630 (m), 538 (m), 487 (m), 463 (w), 445 (vw), 442 (m).

2.3.4. Re[S-$O_2CCHBrCH(CH_3)_2$](CO)$_3$(bpy) (Complex 4S).

Complex 4S was prepared by dissolving 0.050 g (0.090 mmol) of Complex 7 and 0.021 g (0.12 mmol) of *S*-2-bromo-3-methylbutyric acid in 10 mL of dichloromethane. Complex 4S (0.037 g, 0.061 mmol, 68%) was recovered by the addition of 10 mL of MTBE and 15 mL of pentane after 15 min. of stirring. The precipitate of Complex 4S was filtered and washed with two 5 mL portions of MTBE. Anal. Calcd for $C_{18}H_{16}BrN_2O_5Re$: C, 35.65; H, 2.65; N, 4.62. Found: C, 35.43; H, 2.53; N, 4.82. ^1H NMR (d_6-DMSO): 9.04 (m, 2H, bpy), 8.76 (d 8.1 Hz, 2H, bpy), 8.35 (m, 2H, bpy), 7.76 (m, 2H, bpy), 3.74 (d 6.7 Hz, 1H, -CHBr-), 1.50 (octet 6.6 Hz, 1H, -$CHMe_2$), 0.52 (d 6.6 Hz, 3H, CH_3), 0.49 (d 6.7 Hz, 3H, CH_3). ^{13}C-{^1H} NMR (d_6-DMSO): 197.79 (s, CO), 195.14 (S, CO), 171.81 (s, -OC(O)-), 155.88 (s, bpy 2 and 2'), 153.89 (s, bpy 6 or 6'), 153.84 (s, bpy 6 or 6'), 141.01 (s, bpy 4 and 4'), 128.13 (s, bpy 3 or 3'), 128.08 (s, bpy 3 or 3'), 124.56 (s, 5 and 5'), 61.58 (s, -CHBr-), 32.58 (s, -$CHMe_2$), 20.01 (s, CH^3), 19.57 (s, CH^3). IR (ATR cm^{-1}): 3083 (vw), 2973 (w), 2016(s), 1902 (s), 1865 (vs), 1638 (m), 1603 (m), 1497 (vw), 1473 (m), 1444 (m), 1354 (m), 1322 (m), 1245 (w), 1231 (w), 1196 (w), 1176 (w), 1159 (w), 1105 (w), 1076 (w), 1047 (w), 1034 (w), 906 (w), 808 (vw), 770 (s), 731 (m), 720 (m), 676 (vw), 648 (w), 634 (m), 533 (m), 487 (m), 461 (w), 446 (vw), 419 (m).

2.3.5. Re[R-$O_2CCHBrCH(CH_3)_2$]$(CO)_3$(bpy) (Complex 4R).

Complex 4R was prepared by dissolving 0.050 g (0.090 mmol) of Complex 7 and 0.019 g (0.10 mmol) of *R*-2-bromo-3-methylbutyric acid in10 mL of dichloromethane. Complex 4R (0.028 g, 0.046 mmol, 51%) was recovered by the addition of 10 mL of MTBE and 15 mL of pentane after 15 min. of stirring. The precipitate of Complex 4R was filtered and washed with two 5 mL portions of MTBE. Anal. Calcd for $C_{18}H_{16}BrN_2O_5Re$: C, 35.65; H, 2.65; N, 4.62. Found: C, 35.39; H, 2.74; N, 4.46.

2.3.6. Re[$O_2C(CH_2)_4CH_3$]$(CO)_3$(bpy) (Complex 5).

Complex 5 was prepared by combining 0.100 g (0.179 mmol) of Complex 7 and 0.50 mL (4.0 mmol) of hexanoic acid in 5 mL of thf. After 30 min. precipitation of Complex 5 was induced by the addition of 10 mL of MTBE and 10 mL of pentane. The precipitate of Complex 5 (0.087 g, 0.16 mmol, 90%) was collected by filtration and washed with 5 mL portions of MTBE. Anal. Calcd for $C_{19}H_{19}N_2O_5Re$: C, 42.14; H, 3.51; N, 5.17. Found: C, 41.92; H, 3.62; N, 5.24. ^1H NMR (d_6-DMSO): 9.03 (d 5.1 Hz, 2H, bpy), 8.76 (d 8.1 Hz, 2H, bpy), 8.34 (m, 2H, bpy), 7.75 (m, 2H, bpy), 1.63 (t 6.7 Hz, 2H, -(C2)H_2-), 0.89 (p 6.7 Hz, 4H, -(C3)H_2-(C4)H_2-), 0.65 (t 6.7 Hz. 3H, CH_3), 0.61 (m, 2H, -(C5)H_2-). ^{13}C-{^1H} NMR (d_6-DMSO): 199.06 (s, CO), 195.07 (s, CO), 176.82 (s, -C(O)O-), 155.70 (s, bpy), 153.82 (s. bpy), 140.83 (s, bpy), 128.00 (s, bpy), 124.42 (s, bpy), 36.44 (s, C2), 31.03 (s, C3), 25.97 (s, C4), 22.31 (s, C5), 14.23 (s, C6). IR (ATR cm^{-1}): 2924 (w), 2856 (w), 2015 (s), 1894 (s), 1863 (vs), 1625 (m), 1599 (m), 1573 (w), 1492 (w), 1470 (m), 1444 (m), 1369 (m), 1330 (m), 1313 (m), 1252 (m), 1225 (m), 1175 (w), 1156 (vw), 1106 (vw), 1071 (vw), 1047 (vw), 1035 (vw), 969 (vw), 893 (vw), 804 (vw), 763 (s), 731 (m), 662 (m), 649 (m), 629 (vw), 569 (m), 537 (m), 489 (vw), 461 (vw), 444 (vw), 418 (m).

2.3.7. ReCl$(CO)_3$(bpy).

The complex ReCl$(CO)_3$(bpy) was prepared by stirring 0.100 g (0.179 mmol) of Complex 7 with 0.051 g (0.41 mmol) of $CH_3O_2CCH_2NH_2 \cdot HCl$ in 10 mL of dichloromethane for 2 hours. A yellow precipitate of ReCl$(CO)_3$(bpy) (0.070 g, 0.15 mmol, 85 %) formed from the filtration of the mixture into 30 mL of ethyl ether. The product was filtered and characterized by IR and NMR. ^1H NMR (d_6-DMSO): 9.03 (d 5.1 Hz, 2H, bpy), 8.78 (d 8.2 Hz, 2H, bpy), 8.35 (t 7.8 Hz, 2H, bpy), 7.77 (m, 2H, bpy). ^{13}C-{^1H} NMR (d~~6~~-DMSO): 198.25 (s, CO), 190.50 (s, CO), 155.62 (s, bpy), 153.43 (s, bpy), 140.79 (s, bpy), 128.37 (s, bpy), 124.81 (s, bpy). IR (ATR cm^-

^{1}): 2014 (s), 1891 (vs), 1874 (vs), 1744 (s), 1601 (w), 1584 (w), 1496 (m), 1470 (m), 1438 (m), 1423 (m), 1401 (m), 1245 (s), 1140(w), 1059 (m), 956 (w), 900 (m), 879 (m), 773 (vw), 765 (s), 731 (m), 458 (m).

2.3.8. [Re(CO)$_3$(bpy)(NCCH$_3$)]BF$_4$.

The complex [Re(CO)$_3$(bpy)(NCCH$_3$)]BF$_4$ was prepared by treating 0.052 g (0.093 mmol) of Complex 7 suspended in 5 mL of acetonitrile with 30 μL of 7.1 M HBF$_4$·Et$_2$O solution (0.21 mmol). After 15 minutes the resultant solution was added to 25 mL of MTBE. The resultant yellow solid of [Re(CO)$_3$(bpy)(NCCH$_3$)]BF$_4$ was filtered, washed with MTBE, and dried (0.027 g, 0.049 mmol, 52 %). ^{1}H NMR (d3-acetonitrile): 9.05 (m, 2H, bpy), 8.53 (d 8.2 Hz, 2H, bpy), 8.33 td 7.9 and 1.4 Hz, 2H, bpy) 7.75 (ddd 6.8 5.5 and 1.2 Hz, 2H, bpy), 2.07 (s, 3H, CH$_3$). ^{13}C-{^{1}H} NMR (d$_3$-acetonitrile): 193.89 (s, CO), 190.45 (s, CO), 156.10 (s, bpy), 154.01 (s, bpy), 140.99 (s, bpy), 128.18 (s, bpy), 124.42 (s, bpy), 122.52 (s, NCMe), 27.05 (s, CH3). IR (ATR cm-1): 3132 (w), 2945 (w), 2291 (w), 2031 (s), 1908 (s), 1606 (m), 1500 (w), 1476 (m), 1447 (m), 1372 (vw), 1321 (w), 1286 (w), 1247 (w), 1228 (vw), 1167 (w), 1049 (s), 1021 (s), 895 (vw), 811 (w), 771 (s), 749 (w), 732 (m), 662 (w), 643 (m), 624 (m), 537 (m), 520 (m), 482 (s), 462 (w), 433 (vw), 422 (m), 407 (m).

2.4. One-pot preparations of complexes.

2.4.1. Re(O$_2$CCH$_3$)(CO)$_3$(bpy) (Complex 2).

Complex 2 was prepared by combining 0.101 g (0.155 mmol) of Re$_2$(CO)$_{10}$ with 0.049 g (0.31 mmol) of bpy and 8 mL of phenyl acetate in a 50 mL round-bottomed flask. The solution was heated to 140 °C for 24 hours. Upon cooling the reaction mixture was added to 10 mL of MTBE and 25 mL of hexanes. The resultant yellow powder that formed upon stirring (0.112 g, 0.231 mmol, 74.5 %) was recovered by filtration, washed with hexanes, and dried. ^{1}H NMR (d$_6$-DMSO): 9.04 (m, 2H, bpy), 8.74 (d 8.4 Hz, 2H, bpy), 8.34 (td 7.8 and 1.6 Hz, 2H, bpy), 7.76 (ddd, 6.7 Hz, 5.5 Hz, 1.1 Hz, 2H, bpy), 1.39 (s, 3H, acetate). ^{13}C-{^{1}H} NMR (d$_6$-DMSO): 198.99 (s, CO), 194.97 (s, CO), 174.59 (s, -OC(O)-), 155.59 (s, bpy), 153.88 (s, bpy), 140.89 (s, bpy), 128.10 (s, bpy), 124.52 (s, bpy), 23.59 (s, CH3). IR (ATR cm^{-1}). 3082 (vw), 2011 (s), 1874 (vs), 1762 (w), 1623 (m), 1591 (m), 1494 (vw), 1473 (m), 1445 (m), 1368 (s), 1332 (m), 1314 (s), 1285 (w), 1246 (w), 1195 (w), 1161 (w), 1074 (vw), 1048 (vw), 1021 (w), 907 (vw), 817 (vw), 774 (s), 733 (m), 698 (m), 672 (m), 648 (m), 625 (m), 533 (m), 511 (vw), 487 (m), 461 (w), 446 (vw), 423 (m).

2.4.2. Re(O$_2$CCH$_3$)(CO)$_3$(phen) (phen = 1,10-phenanthroline).

This complex was prepared by heating a solution of Re$_2$(CO)$_{10}$ (0.090 g, 0.14 mmol) and phen (0.047 g, 0.30 mmol) in 5 mL of phenyl acetate for 19 hours at 140 °C. Upon cooling, the solution was transferred to 50 mL of hexanes. Stirring induced a precipitate which was filtered, washed with two 5 mL portions of hexanes and dried. A mass of 0.078 g (0.15 mmol, 54 %) of

$Re(O_2CCH_3)(CO)_3(phen)$ was recovered. ^1H NMR (d_6-acetone): 9.55 (dd 5.1 and 1.4 Hz, 2H, phen), 8.97 (dd 8.3 and 1.4 Hz, 2H, phen), 8.34 (s, 2H, phen), 8.15 (dd 8.3 and 5.1 Hz), 1.25 (s, 3H, CH3). ^{13}C-{^1H} NMR (DMSO): 198084 (s, CO), 194.92 (s, CO), 174.65 (s, -OC(O)-), 154.49 (phen), 146.31 (s, phen), 139.94 (s, phen), 130.78 (s, phen), 128.21 (s, phen), 126.86 (s, phen), 23.52 (s, CH3). IR (ATR cm^{-1}): 2010 (s), 1902 (s), 1860 (vs), 1612 (m), 1517 (w), 1425 (m), 1368 (m), 1315 (m), 1222 (vw), 1145 (w), 1093 (vw), 1012 (vw), 932 (vw), 847 (m), 776 (vw), 723 (m), 672 (m), 649 (m), 546 (m), 490 (m), 473 (w).

2.4.3. Re(O₂CPh)(CO)₃(bpy) (Complex 6).

This complex was prepared by heating a solution of $Re_2(CO)_{10}$ (0.489 g, 0.75 mmol) and bpy (0.258 g, 1.65 mmol) in 25 mL of methyl benzoate at 140 °C for 24 hours. Upon cooling to 0 °C 0.455 g (0.831 mmol, 55.4 %) precipitated and was recovered by filtration. Anal. Calcd for $C_{20}H_{13}N_2O_5Re$: C, 43.87; H, 2.39; N, 5.12. Found: C, 43.83; H, 2.44; N, 5.13. ^1H NMR (d_6-DMSO): 9.07 (d 4.8 Hz, 2H, bpy), 8.76 (d 7.9 Hz, 2H, bpy), 8.34 (t 7.6 Hz, 2H, bpy), 7.76 (m, 3H, bpy and *p*-Ph), 7.55 (m, 2H, Ph), 7.24 (m, 2H, Ph). ^{13}C-{^1H} NMR (d_6-DMSO): 198.87 (s, CO), 195.10 (s, CO), 169.82 (s, -OC(O)-), 155.75 (s, aromatic), 153.84 (s, aromatic), 141.05 (s, aromatic), 135.64 (s, aromatic), 130.65 (s, aromatic) 129.03 (s, aromatic), 128.20 (s, aromatic), 128.03 (s, aromatic), 124.55 (s, aromatic). IR (ATR cm^{-1}): 2015 (s), 1908 (vs), 1842 (vs), 1616 (m), 1600 (m), 1581 (m), 1491 (vw), 1472 (m), 1446 (m), 1360 (s), 1341 (s), 1316 (m), 1245 (vw), 126 (w), 1109 (vw), 1070 (w), 1047 (vw), 1021 (w), 837 (w), 813 (vw), 773 (s), 721 (s), 691 (w), 647 (m), 631 (w), 581 (vw), 537 (m), 485 (m), 459 (w).

2.5. NMR tube substitution experiments.

2.5.1. General conditions

Proton NMR substitution experiments were measured at a probe temperature of 295 K. Unless otherwise specified each sample was saturated in rhenium-containing test complex and the undissolved solid was removed by filtration before loading the sample tube. Solutions were maintained in a dark cabinet when the sample was not in the spectrometer. Three solvent systems were tested: a nonprotic system consisting of 1:1 nitrobenzene:d_3-acetonitrile, a protic solvent system consisting of 1:1 d_6-DMSO:d_2-water, and a chloride-rich solvent system consisting of 0.13 M NaCl in 1:1 d_6-DMSO:d_2-water.

2.5.2. Substitution investigations in the three different solvent systems.

A saturated solution of Complex 3, and a solution containing 10.3 mg (0.021 mmol) of Complex 2 dissolved in 1.5 mL of the nonprotic solvent system were measured before and after standing for 24 hours. Complex 1 was not measured in the nonprotic solvent system due to overlap of the carboxylate ligand proton with nitrobenzene resonances. Complexes 1-3 and $ReCl(CO)_3(bpy)$ were each measured before and after standing in the protic solvent system for 24 hours. Only solutions of Complex 2 or $ReCl(CO)_3(bpy)$ were measured before and after standing for 24 hours in the chloride-rich solvent system.

2.5.3. Substitution reactions of Complex 2 in the presence of proton sources.

A solution of 10.3 mg of Complex 2 (0.021 mmol) in 1.5 mL of the nonprotic solvent system was treated with 300 nL of $HBF_4{\cdot}Et_2O$ (0.0021 mmol) solution. The ^{1}H NMR spectrum of the solution was measured before and after standing in solution for 24 hours. The solution was also spiked with 2 μL of glacial acetic acid following the 24 hours measurement and remeasured.

A solution of 7.5 mg of Complex 2 (0.015 mmol) and 1.4 mg of butylammonium tetrafluoroborate (0.0094 mmol) dissolved in 1.5 mL of the nonprotic solvent system (with 10 % of the d_3-acetonitrile replaced with CH_3CN) was measured upon preparing, after 24 hours, and after five days.

A solution of 5.7 mg of Complex 2 (0.012 mmol) and 3.1 mg of butylammonium tetrafluoroborate (0.021 mmol) dissolved in 1.5 ml of the protic solvent system was measured upon mixing and after 24 hours.

A solution of 5.1 mg of Complex 2 (0.011 mmol), 3.3 μL of pyridine (0.041 mmol), and 0.5 mg of pyridinium tetrafluoroborate dissolved in 1.5 mL of the protic solvent system was measured upon mixing, after one day, and after three days of standing.

2.6. Crystal structure of Complex 4R.

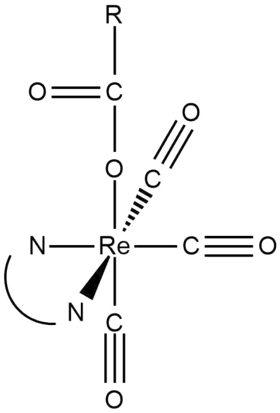
Crystals of 4R were prepared by dissolving 0.015 g of Complex 4R in 5 mL of dichloromethane. A layer of 10 mL of MTBE was added on top of the dichloromethane and a second layer of 15 mL of pentane was added on top of the first two layers. Complete details on the structural determination of Complex 4R are given in the Supporting Material or at CCDC 2332556.

3. Results

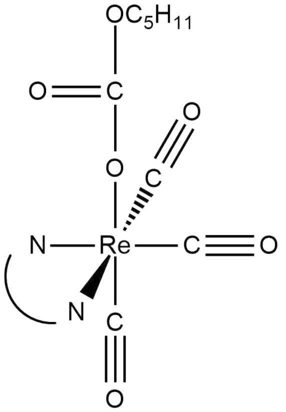
3.1. Synthesis and characterization.

3.1.1. Acidolysis of $Re(O_2COC_5H_{11})(CO)_3(bpy)$.

Six-coordinate rhenium(I) centers (low-spin d^6) are generally inert towards substitution reactions. However, Mandal's method which consists of acidolysis of the pentylcarbonate ligand in Re($O_2COC_5H_{11}$)$(CO)_3$(bpy) (Complex 7) by a carboxylic acid (Equation 2), provides a convenient route to substitute the carboxylate-stabilized complexes in this report (Figure 1) [23]. The acid-promoted decomposition of the alkylcarbonate ligand affords an opportunity to bind a variety of nucleophiles to rhenium(I) tricarbonyl centers (Section 4.1).



Complexes 1-6



Complex 7

R = H (1), CH_3 (2), CHF_2 (3), *S*-$CHBrCH(CH_3)_2$ (4S), *R*-$CHBrCH(CH_3)_2$ (4R), C_5H_{11} (5), or Ph (6)

= 2,2'-bipyridine

Figure 1. Representations of the carboxylate-stabilized complexes in this report and the alkylcarbonate-stabilized complex which was the starting material for acidolysis reactions.

Complex 7, the pentylcarbonate-stabilized starting material for Mandal's Method, was prepared with the initially reported thermal route to that complex [23]. A recent publication describes a faster microwave-assisted route to the pentylcarbonate-stabilized complex along with other alkylcarbonate-stabilized complexes that should work equally well for the preparation of carboxylate-stabilized products [24]. Preliminary work in our lab indicates that other alkylcarbonate-stabilized rhenium(I) tricarbonyl complexes, such as Re($OCO_2C_8H_{17}$)$(CO)_3$(phen) (phen = 1,10-phenanthroline), also produce carboxylate-stabilized rhenium(I) complexes upon reaction with a carboxylic acid (Supporting Material).

3.1.2. Thermolysis of $Re_2(CO)_{10}$ with esters.

While preparing the carboxylate-stabilized rhenium(I) complexes for this study, an apparently generic one-pot method was identified for preparing such complexes through the thermolysis of $Re_2(CO)_{10}$ and a diimine in an appropriate ester solvent. Two ester properties are seemingly necessary for successful thermal transformation of $Re_2(CO)_{10}$, a diimine, and an ester into a carboxylate-stabilized rhenium(I) tricarbonyl complex: 1) a boiling point of 140 ^{o}C or higher and 2) an aromatic ring on at least one side of the ester. Under our experimental conditions of heating a solution of $Re_2(CO)_{10}$ and diimine dissolved in the ester under a pressure of 1 atm, the high boiling ester is required in order to provide sufficient thermal energy to disrupt the dirhenium decacarbonyl starting material. It was also observed that a solution of $Re_2(CO)_{10}$ and diimine in propyl butyrate, an ester which is entirely aliphatic, does not go directly to the expected product, $Re(O_2CCH_2CH_2CH_3)(CO)_3(N\text{-}N)$ (N-N = bpy or phen). Rather than the expected product, a purple solution forms. The purple solution, based upon reports in the literature, likely includes a rhenium-bound radical diimine ligand with one additional electron in its π^* system [27]. When the purple solution is allowed to stand, exposed to air, for several days the solution turns orange and an impure sample of the expected complex, $Re(O_2CCH_2CH_2CH_3)(CO)_3(N\text{-}N)$, can be recovered (Supporting Material). Unlike entirely aliphatic ester solvent/reactants, esters such as methyl benzoate or phenyl acetate, with an aromatic ring on either side of the ester functional group, go cleanly to complexes such as $Re(O_2CCH_3)(CO)_3(bpy)$, $Re(O_2CCH_3)(CO)_3(phen)$, or $Re(O_2CPh)(CO)_3(bpy)$ when reacted with $Re_2(CO)_{10}$ and a diimine.

3.1.3. Spectroscopic characterizations.

All carboxylate-stabilized rhenium(I) tricarbonyl complexes in this report were characterized by ^1H and $^{13}C\text{-}\{^1H\}$ NMR spectroscopy as well as by infrared spectroscopy (Supporting Material). Complexes 1-6 and $Re(O_2CPh)(CO)_3(phen)$, which were either tested for cytotoxicity or are previously unreported complexes, were also characterized by CHN elemental analysis (Experimental Section). The most interesting NMR spectra for the complexes in this report were the ^1H NMR spectra of Complexes 4S or 4R, the chiral complexes. Either chiral complex exhibits a pair of doublet resonances near $\delta = 0.5$ ppm, in the ^1H NMR spectrum, which are separated by 0.027 ppm (at 400 MHz, 0.027 ppm = 10.8 Hz) with $^3J_{HH}$ coupling of 6.7 Hz for the diastereotopic methyl groups of the carboxylate ligand (Figure 2). Further evidence for chirality is seen for the set of bpy protons which are chemically equivalent, as pairs, for equivalent ring positions on the two aromatic rings in an achiral complex such as Complex 2 (Figure 2 Insert A). The ^1H NMR aromatic resonances for two of the four sets of bpy protons on the chiral complexes appear as more complex resonances than are observed for Complex 2 due to the diastereotopic nature of the pairs of bpy protons in Complexes 4S or 4R (Figure 2).

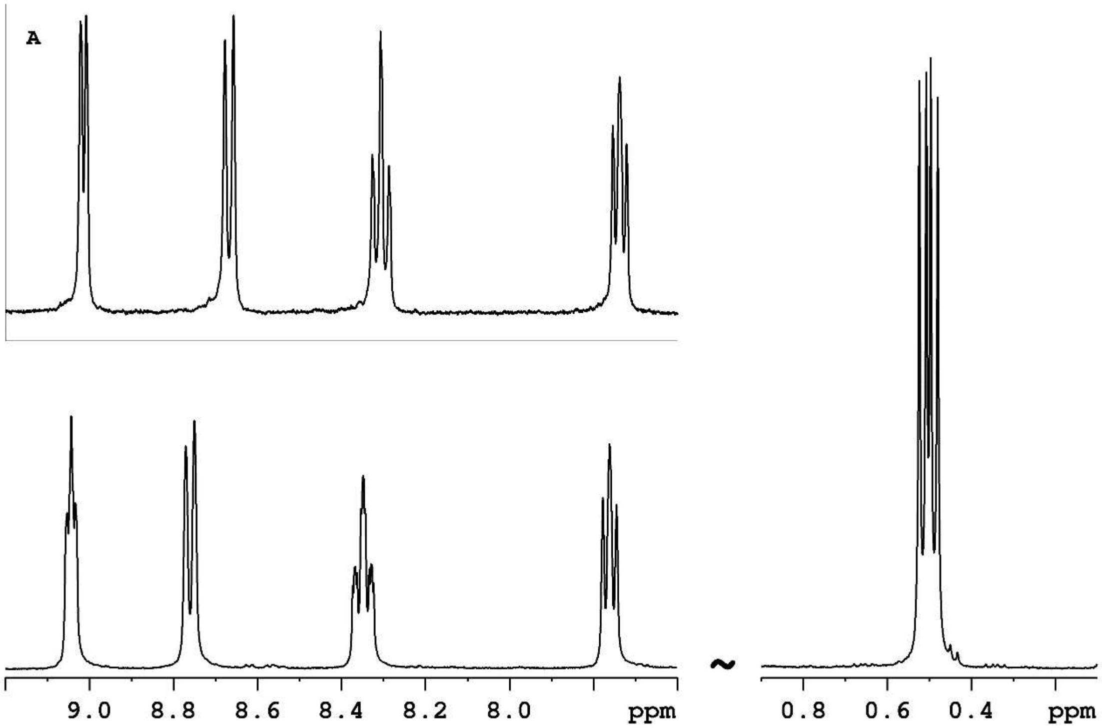


Figure 2. The ^{1}H NMR resonances measured in d_6-DMSO for diastereotopic protons of Complex 4S along with an inset (A) of the nondiastereotopic bpy proton resonances for Complex 2 measured in d_6-DMSO.

3.1.4. X-ray diffraction analysis of Complex 4R.

The complexes in this report were prepared to examine the effect that variation in carboxylate identity has on the cytotoxicity of the resultant complexes. Complexes 4S and 4R were specifically prepared to compare the effect on cytotoxicity for a pair of enantiomers when the chiral center is located on the carboxylate ligand. It has previously been shown that a chiral center on a diimine ligand produces differing levels of cytotoxicity for the two enantiomers [21]. Given that complexes 4S and 4R were prepared using enantiotopically pure versions of *S*- or *R*-$(CH_3)_2CH(Br)CO_2H$, it was assumed that the complexes would also be enantiomerically pure. Confirmation of the enantiomeric purity and absolute structure of Complex 4R was made by a single crystal X-ray diffraction analysis of the material.

Crystals of 4R were prepared by dissolving 0.015 g of Complex 4R in 5 mL of dichloromethane. A layer of 10 mL of MTBE was added on top of the dichloromethane and a second layer of 15 mL of pentane was added on top of the first two layers. After several days, crystals of 4R were harvested. Most of the recovered crystals were twinned but a single crystal of approximate dimensions 0.21 mm × 0.15 mm × 0.08 mm and without significant twinning was used for the single crystal X-ray diffraction analysis (see Supporting Material for all crystallographic details or CCDC 2332556). Analysis of a data set consisting of 105,868 reflections produced a monoclinic unit cell with a space group of $P2_1$ containing two independent molecules of 4R (Figure 3). The Flack parameter for the model is 0.985(6) indicating good enantiomeric purity and a lack of twinning. The two molecules of 4R within the unit cell are nearly related by inversion symmetry, except for the chiral carboxylate ligands which maintain their stereochemistry. The most significant violation of this pseudo-symmetry relationship between the two unique molecules in the crystal structure of 4R involves a rotation of isopropyl groups of the two carboxylate ligands. The bond distances and bond angles are nearly identical for the two unique molecules, but the torsion angles differ for the two carboxylate ligands (O-C-C-C of +143° *versus* -70°) (Figure 4). In this way, the unique chirality and efficient crystal packing in 4R crystal structure is maintained (Table 1 and Supporting Material). The Re-O bond distances of about 2.166 Å are within the range of distances previously observed for three other carboxylate-stabilized rhenium(I) tricarbonyl complexes supported by bpy [28-30]. Within the unit cell of 4R, as presented in Figure 2, the equatorial planes of the two molecules (as defined by the bpy ligands) are arranged nearly parallel to one another. Adjacent molecules pack with repeated π···π stacking interactions in a 1D fashion along the crystallographic a-axis (see unit cell packing diagram in Supporting Material). The Br atoms of the 4R molecules in each 1D chain are oriented in the same direction. Although not related by any crystallographic symmetry, adjacent 1D chains in the b-axis direction have Br atoms oriented in approximately the opposite direction. Also, along the a-axis are found methyl CH···Br interactions with separations that are approximately equal to the respective van der Waals radii sum. Along the crystallographic c-axis direction the two molecules orient with axial carbonyl ligands nearly parallel to one another. Each axial carbonyl ligand orients with its oxygen atom directed towards the centroid of the equatorial bipyridine ligands of the adjacent 4R molecule. The packing of the two 4R molecules also allows the alkyl groups of the carboxylate ligands to make short methyl CH···O contacts to the carbonyl group of a nearby 4R complex along the crystallographic b-axis. The carboxylate alkyl groups of Complex 4R pack in a manner that minimizes the approach of the electronegative bromine atoms towards one another.

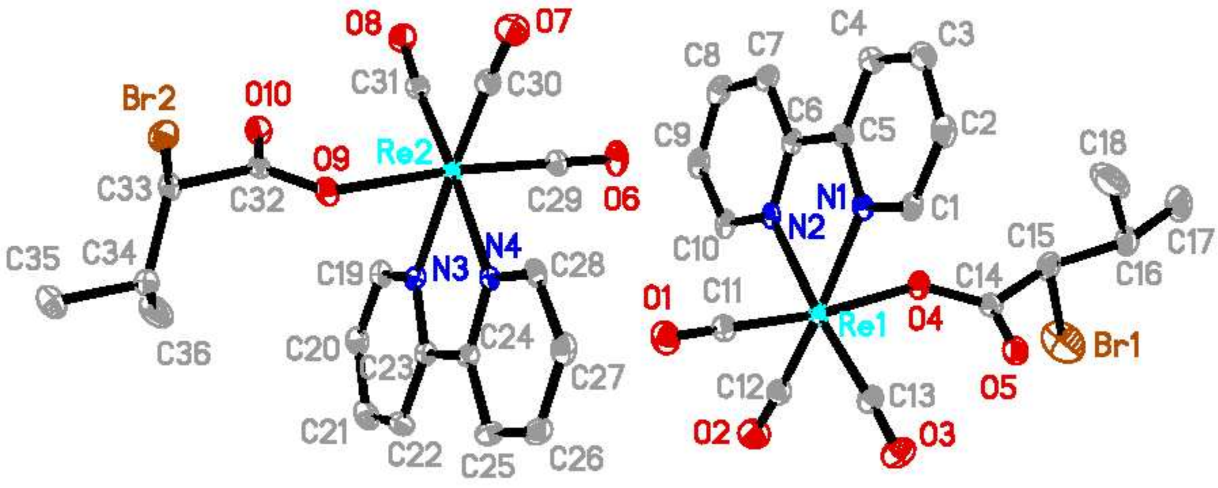


Figure 3. An ORTEP representation of the two molecules of Complex 4R within the unit cell.

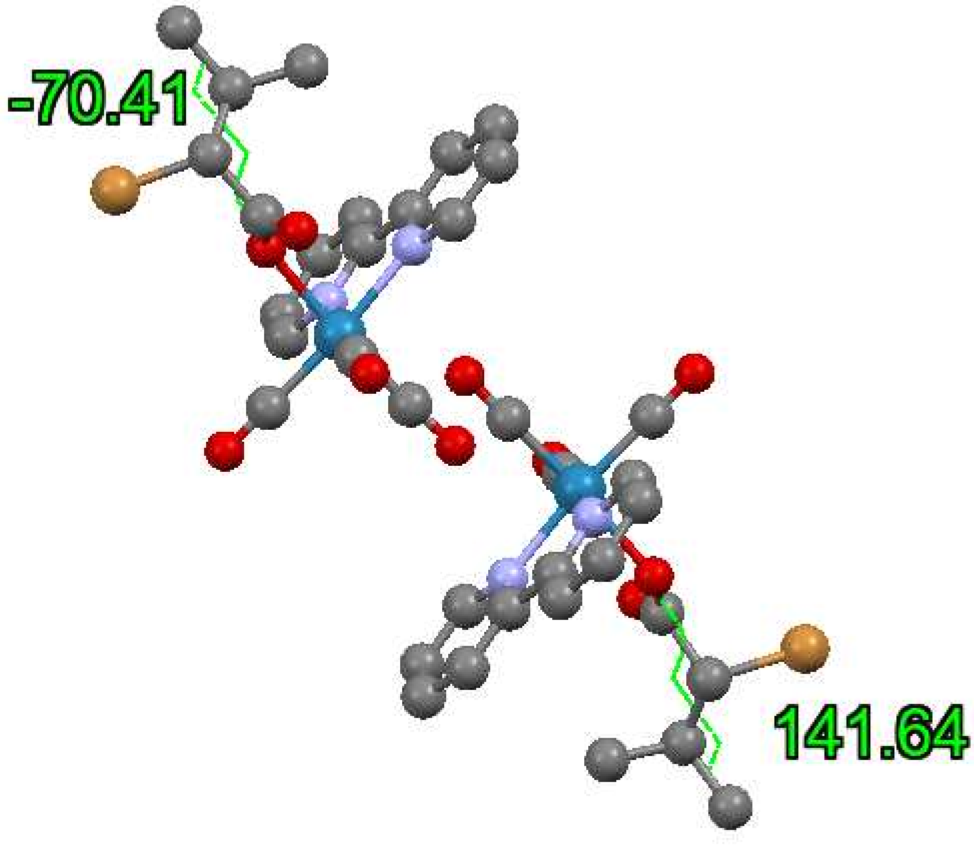


Figure 4. A representation of the two different torsion angles which make the two molecules within the unit cell independent.

Re1-O4	2.163(3)	Re2-O9	2.168(3)
Re1-N1	2.173(4)	Re2-N3	2.167(4)
Re1-N2	2.168(4)	Re2-N4	2.178(4)
Re1-C11	1.924(4)	Re2-C29	1.903(4)
Re1-C12	1.934(5)	Re2-C30	1.917(4)
Re1-C13	1.940(5)	Re2-C31	1.926(4)

Table 1. Selected bond distances (Å) for the two molecules of 4R within the unit cell.

3.2. Cytotoxicity.

A goal of this work was to survey the potential relationship between the carboxylate ligands in a series of $Re(O_2CR)(CO)_3(bpy)$ complexes and the effect of the complexes on the proliferation of HSC-2 cells and HF-1 normal human cells. Several carboxylate-stabilized rhenium(I) complexes have already been tested for their effect on several different cancer cell lines [6,15,18,23]. To the best of our knowledge, however, this is the first examination of the antiproliferative properties of carboxylate-stabilized rhenium(I) tricarbonyl complexes using an oral cancer cell line. Figure 5 presents the HSC-2 cell viability test results for Complexes 1-3. Cell viability plots for cis-Platin with HSC-2 and HF-1 and Complexes 1-3 with HF-1 cells can be found in the Supporting Material. Table 2 presents IC_{50} values for Complexes 1-3 and *cis*-Platin versus the two cell lines. Complexes 4S, 4R, 5, and 6 did not offer sufficient solubility in PBS to measure their impact on cell viability [14]. Complexes 1-3 proved to be marginally more cytotoxic towards the HSC-2 cells than towards the normal HF-1 cells. Complexes 1 and 2 were also marginally more cytotoxic towards HSC-2 than was *cis*-Platin (Table 2). Complexes 1 and 2 were more cytotoxic towards HF-1 than was *cis*-Platin.

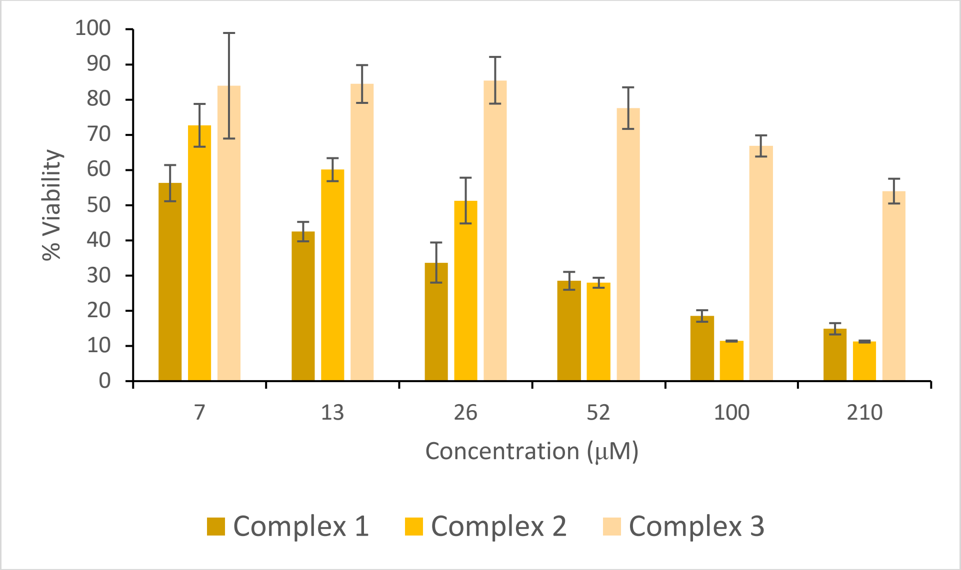


Figure 5. Viability of HSC-2 cells with respect to different concentrations of Complexes 1-3. Error bars represent +/- one standard deviation.

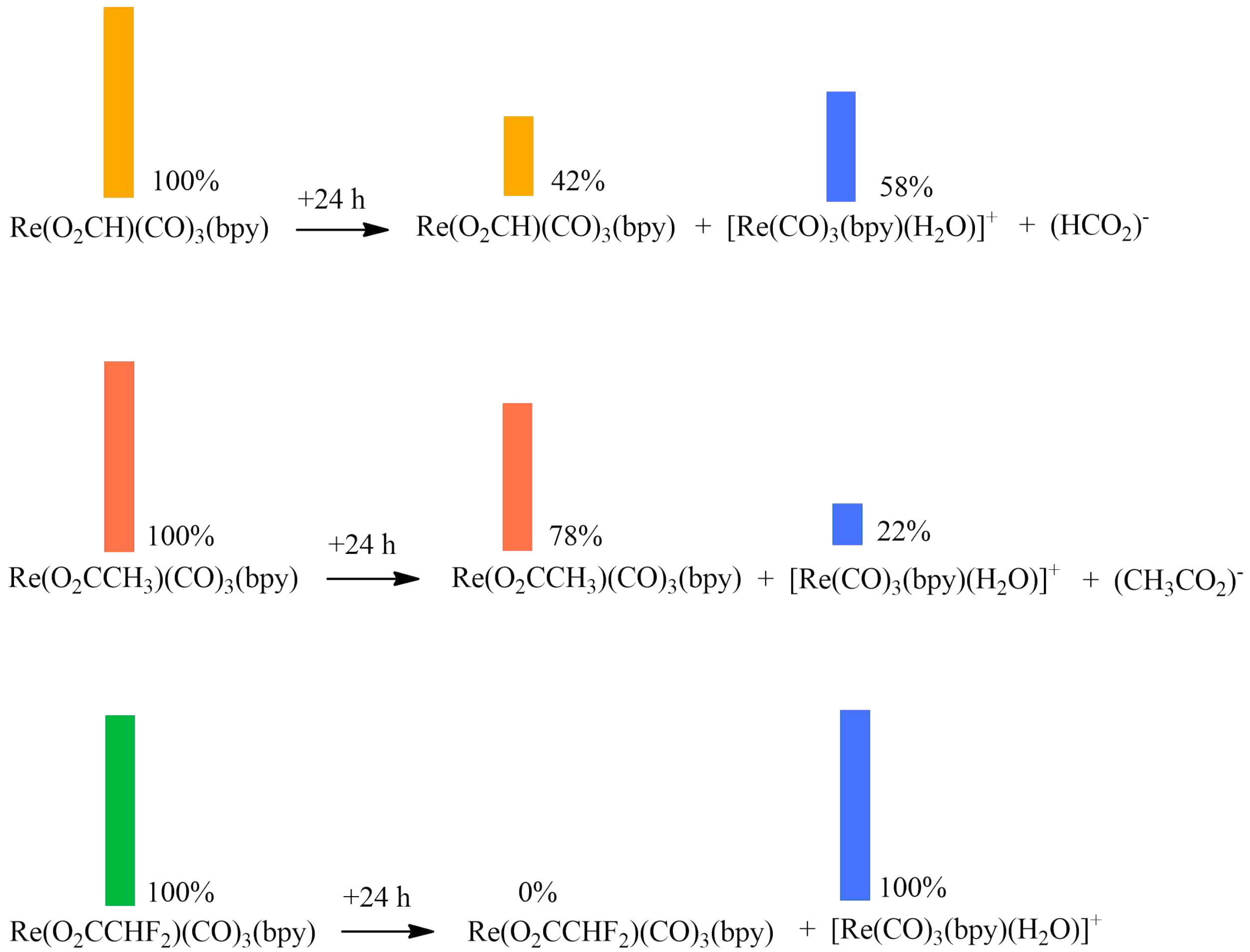
Complex	**Carboxylate Ligand**	**IC_{50} (µM) HSC-2**	**IC_{50} (µM) HF-1**
1	CHO_2^-	19 ± 5.7	>210
2	$CH_3CO_2^-$	26 ± 6.5	160
3	$CHF_2CO_2^-$	>210	>210
cis-Platin	-	48 ± 4.4	>250

Table 2. Experimentally determined IC_{50} values for Complexes 1-3 and *cis*-Platin on both cell lines. IC_{50} values were determined using a polynomial regression.

3.3. Carboxylate ligand substitution.

3.3.1. Carboxylate ligand substitution in nonprotic and protic solvent systems with and without added protons.

Carboxylate-stabilized rhenium(I) complexes are reported to undergo substitution of the carboxylate ligand by water in aqueous systems (Equation 3), especially in the presence of perchloric acid [25], or by chloride ions in a system with high chloride ion concentration [26] despite having six-coordinate, low-spin d^6, rhenium centers [18]. Given the observed cytotoxicity differences for Complexes 1-3 (Table 2) and a report that points towards the water-substituted cation $\{Re(CO)_3(bpy)(H_2O)]^+$ as the active cytotoxic form [9], an NMR study of carboxylate ligand substitution was undertaken to try to better understand the differences in cytotoxicity among the studied complexes. Proton NMR studies of the stability of selected complexes were performed over the course of 24 hours (the length of time used for treatment of cells with the complexes in viability tests) or more. NMR studies of carboxylate ligand substitution were carried out in three different solvent systems; a nonprotic system of 1:1 nitrobenzene:d_3-acetonitrile, a protic system of 1:1 d_6-DMSO:d_2-water, and a 1:1 d_6-DMSO:d_2-water solution which includes 0.13 M NaCl similar to the concentration of chloride ions found in PBS. The nonprotic system was selected because: 1) the tested complexes showed good solubility in the system, 2) d_3-acetonitrile provided a deuterium resonance for locking and shimming the spectrometer, 3) the solvent system supports ionic substances such as might occur if dissociation of a carboxylate ligand from the complex occurs [31], and 4) acetonitrile provides a high concentration reactant that may trap any putative 16 electron rhenium intermediate that forms in solution [32]. At ambient temperature, Complexes 2 and 3 were stable in the nonprotic solvent system for 24 hours (Supporting Material). Complex 1 was not tested in the nonprotic solvent system. In the protic solvent system the resonances of the rhenium-bound carboxylate ligand for Complexes 1- 3 decreased over time while free carboxylate resonances appeared (Supporting Material and Scheme 1). Complex 2 remained mostly unsubstituted (78 % by ^1H NMR integration), Complex 1 was somewhat more substituted (42 % unsubstituted), and Complex 3 was completely substituted within 24 hours in the protic solvent system. The amount of substitution, after 24 hours in the protic system corresponds well with the different K_b values of the carboxylate ligands ($K_{b(formate)} = 5.6 \times 10^{-11}$ [33], $K_{b(acetate)} = 5.8 \times 10^{-10}$ [33], $K_{b(difluoroacetate)} = 1.7 \times 10^{-13}$ [34]). Relatedly, it was also found that the chloro ligand of $ReCl(CO)_3(bpy)$ undergoes substitution in the protic solvent system with 82 % conversion of the complex to, presumably, $[Re(CO)_3(bpy)(H_2O)]^+$ in 24 hours [35].



Scheme 1. Proton NMR observations of carboxylate ligand substitution by water after 24 hours in the protic solvent system.

The effect of acid on acetate ligand substitution for Complex 2 was examined further. When a solution of Complex 2 dissolved in the nonprotic system was treated with 0.10 mole equivalents of $HBF_4 \cdot Et_2O$ solution a stoichiometric amount of acetic acid was formed (Figure 6 and Equation 4). The acetic acid product was identified by spiking the reaction mixture with a sample of glacial acetic acid. The reaction was essentially quantitative. The initial spectrum acquired after mixing the reagents includes a small resonance at 2.45 ppm (Figure 5) which disappears as bound acetate is converted to acetic acid. This small resonance may correspond to a protonated carbonyl analog, $\{Re[OC(O\underline{H})CH_3](CO)_3(bpy)]^+$, to the intermediate observed by IR spectroscopy when $Re(pzCO_2)(CO)_3(bpy)$ was treated with perchloric acid in the solvent acetonitrile [25].

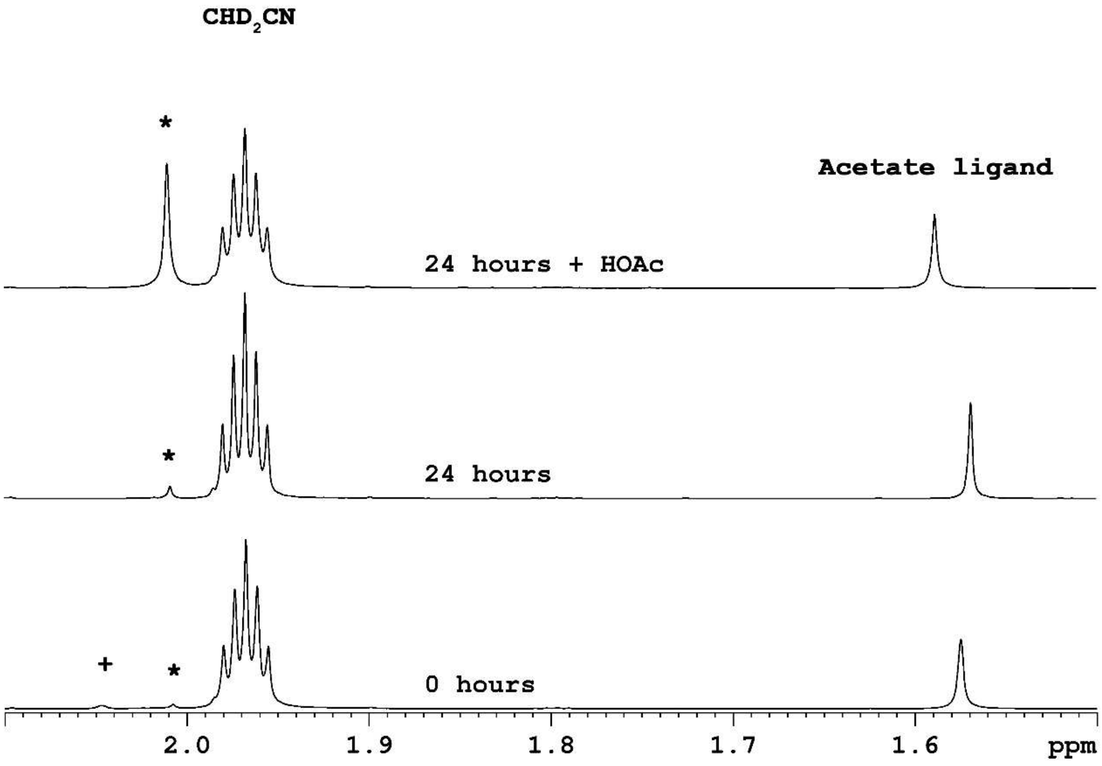


Figure 6. The ^{1}H NMR observation of the stoichiometric transformation of bound acetate to free acetic acid by the addition of 0.10 mole equivalents of $HBF_4 \cdot Et_2O$ solution to a solution of Complex 2 in 1:1 nitrobenzene:d_3-acetonitrile. The resonances marked with * arise from the protons of acetic acid and the resonance marked with + likely arises from the methyl protons of $\{Re[OC(OH)CH_3(CO)_3(bpy)\}^+$.

Equation 4:

$$Re(O_2CCH_3)(CO)_3(bpy) + HBF_4 + CD_3CN \rightarrow [Re(CO)_3(bpy)(NCCD_3)]BF_4 + CH_3CO_2H$$

A carboxylate ligand substitution reaction in the protic NMR solvent mixture was examined with butylammonium tetrafluoroborate as the added proton source. When this experiment was performed without butylammonium tetrafluoroborate (Section 3.3.1.), 22 % of Complex 2 was converted into $[Re(CO)_3(bpy)(H_2O)]^+$ and free acetate ions within 24 hours. When the same solvent mixture contained both Complex 2 and butylammonium tetrafluoroborate, 46 % of Complex 2 was converted into $[Re(CO)_3(bpy)(H_2O)]^+$ and free acetate ions (Figure 7). No change was observed for resonances associated with butylammonium ions during the experiment. For the substitution of acetate by water, for Complex 2 in the protic solvent system, butylammonium cations serve as a catalyst for the substitution reaction.

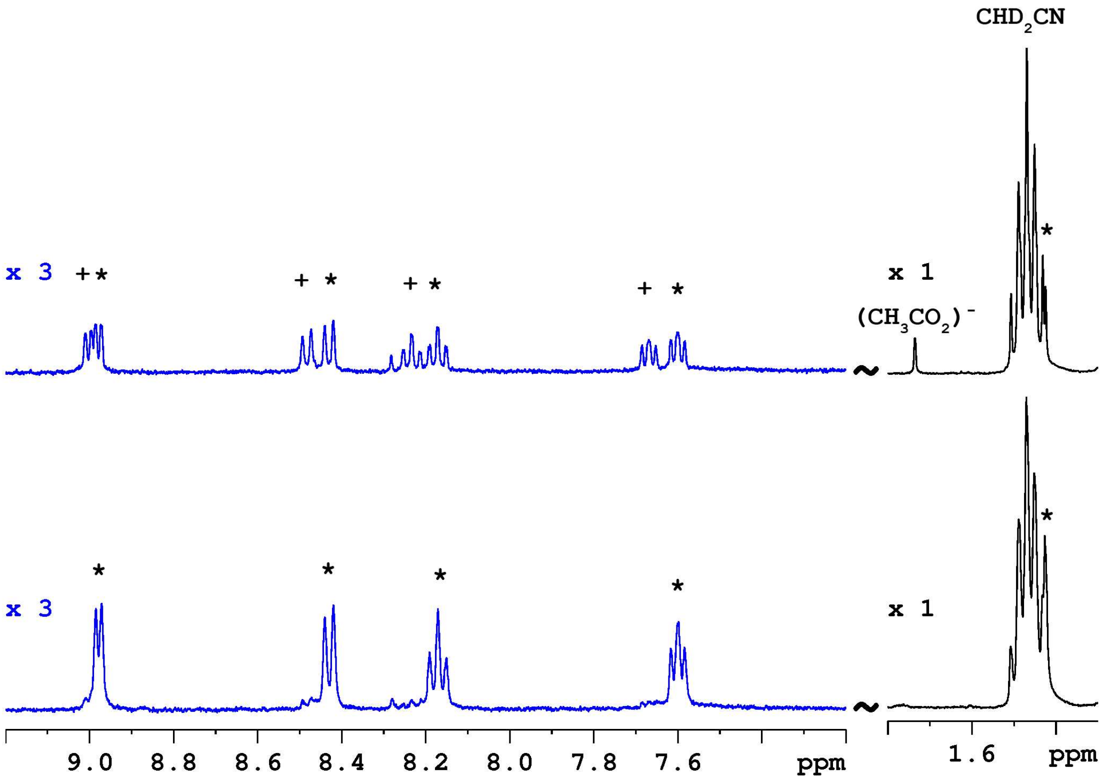


Figure 7. Proton NMR resonances from Complex 2 in a solution of 1:1 d_6-DMSO:d_2-water which contains butylammonium tetrafluoroborate before (lower traces) and after (upper traces) standing for 24 hours. The resonance of the bound acetate ligand protons (~1.42 ppm) overlaps with a resonance from the butylammonium ion. The resonances marked with * arise from Complex 2 and the resonances marked with + arise from $[Re(CO)_3(bpy)H_2O)]^+$.

A second acetate ligand substitution reaction for Complex 2 was examined in a protic solvent system using a combination of pyridinium tetrafluoroborate and pyridine. The reaction converted 40 % of the rhenium bound acetate into free acetate in 24 hours (Figure 8). The reaction also produced a set of three ^{1}H NMR resonances for the furthest downfield bpy pair of protons. The three downfield ^{1}H NMR resonances correspond to bpy protons of: 1) the Complex 2 starting material, 2) the cation $[Re(CO)_3(bpy)(H_2O)]^+$, and 3) the cation $[Re(CO)_3(bpy)(py)]^+$ [36]. By integration of the resonances, production of the $[Re(CO)_3(bpy)(py)]^+$ cation corresponds to only 2 % of the total rhenium content of the sample after 24 hours but increases to 29 % of rhenium in the sample after 72 hours. The appearance of a resonance which assigned to the $[Re(CO)_3(bpy)(py)]^+$ cation and the increasing percentage of rhenium contributing to that resonance indicates that nitrogenous bases can compete effectively with water for a binding site at bpy-stabilized rhenium(I) tricarbonyl centers.

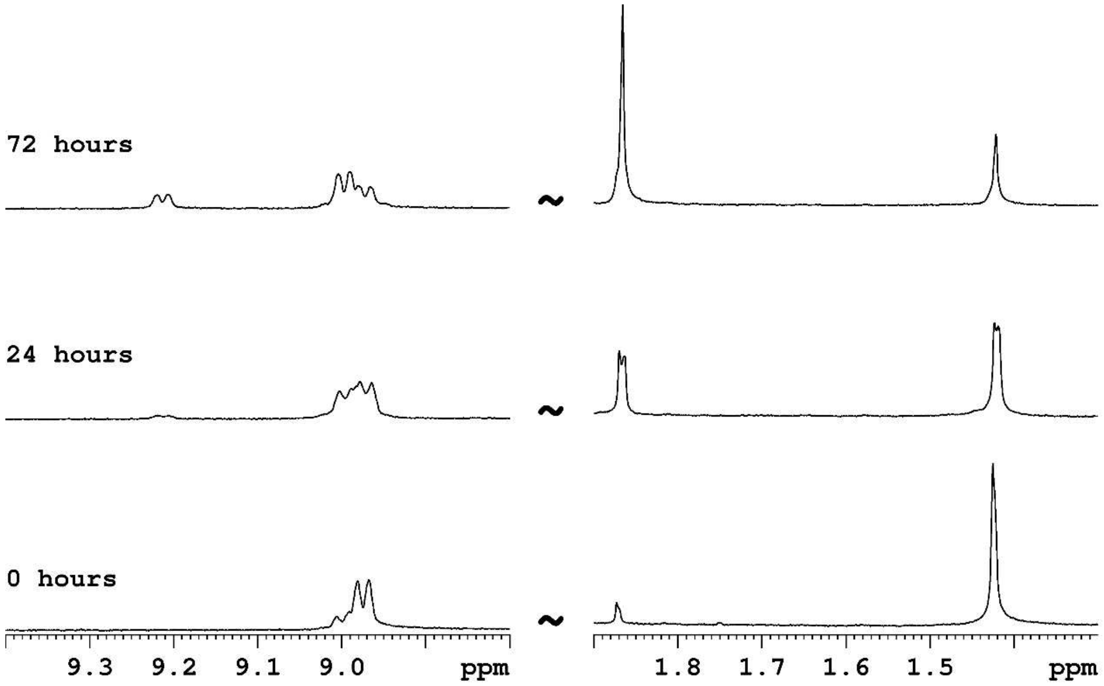


Figure 8. The ^{1}H NMR of rhenium-bound acetate (1.42 ppm) and free acetate (1.87 ppm) resonances along with the furthest downfield resonance for a pair of bpy protons {$Re(O_2CCH_3)(CO)_3(bpy)$ = 8.87 ppm, $[Re(CO)_3(bpy)(H_2O)]^+$ = 9.0 ppm, and $[Re(CO)_3(bpy)(py)]^+$ = 9.22 ppm}as measured in 1:1 d_6-DMSO:d_2-water upon mixing (lowest trace), 24 hours after mixing (middle trace), and 72 hours after mixing (upper trace).

3.3.2. Ligand substitution in a chloride-rich environment.

NMR substitution experiments were also performed on saturated solutions of Complex 2 or $ReCl(CO)_3(bpy)$, in the protic solvent system, with 0.13 M NaCl added in order to expose the complexes to a chloride concentration similar to that found in PBS. Substitution of the acetate ligand of Complex 2 once again occurred slowly over 24 hours with 36 % conversion from bound acetate to free acetate. {The greater amount of substitution compared with the simple protic solvent system (Section 3.3.1) is consistent with the increase in ionic strength for the test solution.} The products for the acetate substitution of Complex 2 were monitored at the bpy proton resonances (Figure 9). The primary product of substitution for Complex 2 is $ReCl(CO)_3(bpy)$. The $ReCl(CO)_3(bpy)$ was identified conclusively by combining the test solution with a second chloride-rich saturated solution of $ReCl(CO)_3(bpy)$ and measuring a portion of the combined solutions. A second product in the test solution is the cytotoxic cation $[Re(CO)_3(bpy)(H_2O)]^+$ (Equation 6). The complex $ReCl(CO)_3(bpy)$ also undergoes substitution in the chloride-rich solution with 33 % conversion of the complex to the water-stabilized cation $[Re(CO)_3(bpy)(H_2O)]^+$. The conversions of Complexes 1-3 and $ReCl(CO)_3(bpy)$ into the water-stabilized cation $[Re(CO)_3(bpy)(H_2O)]^+$ in the protic and in the chloride-rich solvent systems are summarized in Table 3.

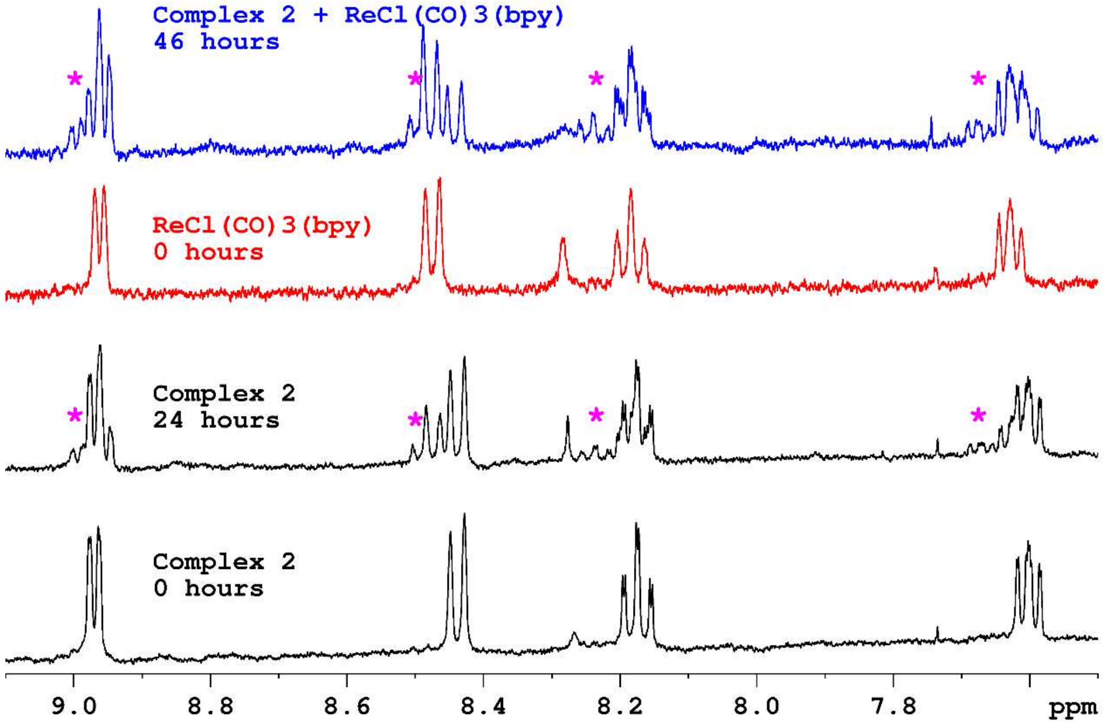


Figure 9. The bpy region of the ^1H NMR spectrum for Complex 2 in d_6-DMSO:d_2-water with 0.13 M Cl^- ions upon mixing (bottom trace), 24 hours after mixing (next to bottom trace), a sample of $ReCl(CO)_3(bpy)$ in the same solvent system (red trace) and a portion of the Complex 2 solution, after 46 hours, combined with fresh $ReCl(CO)_3(bpy)$ solution (blue trace). The resonances marked with an asterisk correspond to the bpy resonances for the cation $[Re(CO)_3(bpy)(H_2O)]^+$ in the same solvent system.

Equation 6

$$\mathrm{Re(O_2CCH_3)(CO)_3(bpy)} + x\,\mathrm{Cl^-} + y\,\mathrm{H_2O} \leftrightarrow$$

$$x\,\mathrm{ReCl(CO)_3(bpy)} + y\,\mathrm{[Re(CO)_3(bpy)(H_2O)]^+} + (x+y)\,\mathrm{(CH_3CO_2)^-}$$

Solvent System	**Complex 1**	**Complex 2**	**Complex 3**	**$ReCl(CO)_3(bpy)$**
Protic	42	22	100	82
Chloride-rich	*	36	*	33

Table 3. Percent conversion of complexes, in either of two solvent systems, into the cation $[Re(CO)_3(bpy)(H_2O)]^+$ during 24 hours. *Not measured.

4. Discussion.

4.1. Acidolysis reactions.

Treatment of Complex 7 with carboxylic acids proved to be a simple route to the complexes of interest for this study. Preliminary studies of acidolysis reactions involving Complex 7 or its 1,10-phenanthroline analog with acids other than carboxylic acids such as the tetrafluoroborate salt of protonated 2-hydroxy-6-methylpyridine. the chloride salt of the protonated methyl ester of glycine, or phenylsulfonic acid provided a mix of results (Supporting Material). The reactant tetrafluoroborate salt of protonated 2-hydroxy-6-methylpyridine did not produce any sample of a pure compound while the $(H_3NCH_2COOCH_3)Cl$ reactant produced pure samples of the complex $ReCl(CO)_3(bpy)$ (Supporting Material) and phenylsulfonic acid produced the commonly used synthetic intermediate $Re(OSO_2Ph)(CO)_3(phen)$. Attempts to prepare amino acid-stabilized rhenium(I) tricarbonyl complexes through acidolysis of alkylcarbonate-stabilized rhenium(I) starting material proved unsuccessful in our hands. The zwitterion form of glycine proved unreactive towards Complex 6 while the hydrochloride salt of glycine led to recovery of $ReCl(CO)_3(bpy)$. Acidolysis using a stoichiometric amount of $HBF_4 \cdot Et_2O$ solution to a suspension of Complex 6 in acetonitrile proceeds cleanly to the solvento complex $[Re(CO)_3(bpy)(NCCH_3)](BF_4)$ (Supporting Material) [32]. Some productive acidolysis reactions of $Re(O_2COC_5H_{11})(CO)_3(bpy)$, beyond reactions with carboxylic acids, are summarized in Scheme 2.

$$+ \mathrm{(H_3NCH_2CO_2CH_3)Cl} \rightarrow \mathrm{ReCl(CO)_3(bpy)} + \mathrm{CO_2} + \mathrm{C_5H_{11}OH} + \mathrm{H_2NCH_2CO_2CH_3}$$

Complex 7 $+ \mathrm{PhSO_3H} \rightarrow \mathrm{Re(OSO_2Ph)(CO)_3(bpy)} + \mathrm{CO_2} + \mathrm{C_5H_{11}OH}$

$$+ \mathrm{HBF_4} + \mathrm{MeCN} \rightarrow \mathrm{[Re(CO)_3(bpy)(NCMe)]BF_4} + \mathrm{CO_2} + \mathrm{C_5H_{11}OH}$$

Scheme 2. Some acidolysis reactions of $Re(O_2COC_5H_{11})(CO)_3(bpy)$ (Complex 7).

4.2. One-pot synthesis.

The most intriguing aspect of the one-pot preparation of carboxylate-stabilized rhenium(I) tricarbonyl complexes supported by a diimine ligand is the need to have an aromatic ring present on the reactant ester even though that aromatic ring can occur on either side of the functional group. The purple solution that forms when $Re_2(CO)_{10}$, a diimine, and an aliphatic ester are heated seems to indicate that aliphatic ester reaction systems lack the ability to oxidize rhenium from Re(0) to Re(I). The aromatic ring of the esters which contain such a ring may serve as a substrate for reductive coupling or some other such transformation that allows for the oxidation of the starting Re(0) centers to Re(I) centers in carboxylate-stabilized products such as $Re(O_2CCH_3)(CO)_3(bpy)$ or $Re(O_2CPh)(CO)_3(bpy)$. Given an appropriate ester, the one-pot synthetic route to carboxylate-stabilized rhenium(I) tricarbonyl complexes may prove to be a more convenient route to such complexes compared with the two step alternative routes involving acidolysis of an alkylcarbonate-containing complex [23], the carboxylic acid induced substitution of bromide in the complex $ReBr(CO)_3(phen)$ [4], or the three step alternative which uses a silver salt to remove a halide ligand from rhenium [8,20,37].

4.3. Carboxylate substitution and differential cytotoxicity.

The NMR substitution experiments described above do not completely reflect the conditions for an antiproliferation test. The protic solutions used in the NMR experiments included 50 % DMSO to achieve sufficient solubility for NMR measurements. The DMSO could potentially solvate neutral complexes in solution in a fashion that might assist the entry of neutral rhenium-containing complexes into the cells. Additionally, chloride-rich solution (0.13 M Cl^-), devoid of phosphate, containing 50 % DMSO rather than 100 % water was used to examine substitution behavior in a chloride-rich environment. If the NMR experiments adequately reflect the conditions for the *in vitro* antiproliferation tests then the differences in cytotoxicity observed for Complexes 1-3 appear to mostly reflect differences in K_b values for the carboxylate ligands. Acetate and formate, the stronger carboxylate bases among the three complexes form the most stable complex with rhenium under conditions that may reflect the extracellular environment. The lesser amount of substitution for Complexes 1 and 2, outside of the cell, suggests that the size of the concentration gradient between the extracellular fluid and the cytosol may be an important factor in the cytotoxicity of these complexes. The greatest concentration gradient exists for the initial complex which is placed into the test solution (Complexes 1, 2, or 3). Complexes 1 and 2, which remain in their initial forms for the longest time will have the greatest driving force for entry into the cells.

Another factor which could contribute to the greater cytotoxicity of Complexes 1 and 2, compared with Complex 3, is the relatively smaller amount of the complex $ReCl(CO)_3(bpy)$ that will form in the chloride-rich extracellular environment. The longer that a carboxylate ligand of a complex remains bound to rhenium in the extracellular environment, the smaller is the amount of $ReCl(CO)_3(bpy)$ that is made. While the complex $ReCl(CO)_3(bpy)$ has been found to be cytotoxic towards MCF7 breast cancer cells, PC3 prostate cancer cells, and H522 lung cancer cells [23]; it also has poor solubility in PBS (Supporting Material). The NMR substitution experiments reported above suggest that the complex $ReCl(CO)_3(bpy)$ should indeed be cytotoxic through the formation of the cation $[Re(CO)_3(bpy)(H_2O)]^+$. The formation of $ReCl(CO)_3(bpy)$ on the route to $[Re(CO)_3(bpy)(H_2O)]^+$, however, is unlikely to benefit the cytotoxicity of the original carboxylate-stabilized complexes. Furthermore, given the low solubility of $ReCl(CO)_3(bpy)$ in a chloride-rich environment, the formation of $ReCl(CO)_3(bpy)$ runs the risk of precipitating some of the rhenium-containing material from the extracellular fluid for *in vitro* cytotoxicity tests.

Results of the NMR substitution experiments in the protic solvent system with added butylammonium tetrafluoroborate or with added pyridinium tetrafluoroborate may relate to the fate of the neutral complexes that enter the cytosol. Protonated amine groups on proteins apparently should be able to serve as catalysts for the substitution of rhenium-bound carboxylate by water in the cytosol. For Complexes 1 and 2, the more substitutionally inert complexes, the cationic cytotoxin $[Re(CO)_3(bpy)(H_2O)]^+$ should form more rapidly in the cytosol, with its smaller chloride ion concentration and potential catalytic alkylammonium ions, than in the extracellular fluid (Equation 3). The NMR substitution experiment with Complex 2, in the protic solvent system, with pyridine and pyridium ions, suggests that the most thermodynamically stable form for rhenium in the cytosol may include a bond between rhenium and an aromatic amine. Previous work has shown that rhenium(I) tricarbonyl diimine centers form stable complexes with 9-ethylguanine [38]. In many respects, the NMR substitution results in this report align with the suggestion that carboxylate-stabilized rhenium(I) tricarbonyl complexes supported by a diimine ligand are cytotoxic through loss of the carboxylate ligand and eventual binding of rhenium to a nitrogen center within the cell [9].

5. Concluding remarks

Assuming the NMR substitution experiments above reflect *in vitro* behavior with cells, carboxylate-stabilized rhenium(I) tricarbonyl complexes, especially Complexes 1 and 2, provide an opportunity to introduce a neutral rhenium-containing complex into the cytosol of a cell. Once in the cytosol the rhenium-containing complex is seemingly able to transform into a reactive water-stabilized intermediate through proton-catalyzed release of the anionic carboxylate ligand into the cytosol. Thus, the *in vitro* behavior of Complexes 1 and 2 reflect the first step of the *in vivo* behavior of *cis*-Platin. The *in vivo* behavior of cis-Platin includes the substitution of an anionic chloride ligand from neutral *cis*-$PtCl_2(NH_3)_2$, by water, as *cis*-Platin moves from the chloride-rich extracellular fluid into the lower chloride concentration cytosol [39].

Declaration of competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Supporting Material

Supporting Material for this article can be found online at

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