Supplementary Material (ESI) for Chemical Communications


# Photoinduced CO release, cellular uptake, and cytotoxicity of a tris(pyrazolyl)methane manganese tricarbonyl complex

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## Supporting Information

## Experimental section

**Materials and methods.** Reactions were carried out in oven-dried Schlenk glassware under an atmosphere of pure nitrogen when necessary and protected from light by wrapping the apparatus in aluminium foil. Solvents were dried over molecular sieves and degassed prior to use. All chemicals were obtained from commercial sources and used without further purification. NMR spectra were recorded on Bruker DPX 200 and DRX 400 spectrometers ($^{1}$H at 200.13 and 400.13 MHz, respectively; $^{13}$C at 50.33 and 100.62 MHz). Chemical shifts $\delta$ in ppm indicate a downfield shift relative to tetramethylsilane (TMS) and were referenced against the signal of the solvent.[1] Coupling constants *J* are given in Hz. Individual peaks are marked as: singlet (s), doublet (d), triplet (t) quartet (q), or multiplet (m). Mass spectra were measured on a VG Autospec (EI, FAB) or Bruker Esquire 6000 (ESI) instrument, only characteristic fragments are given. The solvent flow rate for ESI measurements was 4 $\mu$l·min$^{-1}$ with a nebulizer pressure of 10 psi and a dry gas flow rate of 5 l·min$^{-1}$ at a dry gas temperature of 300 °C. 3-Nitrobenzyl alcohol (3-NBA) was used as the matrix for the FAB measurements. IR spectra were recorded on a Bruker Tensor 27 IR spectrometer equipped with a Pike MIRacle Micro ATR accessory. The elemental composition of the compounds was determined with a VarioEL analyzer from Elementar Analysensysteme GmbH. UV/Vis spectra were recorded on a Varian Cary 100 instrument in 1 cm quartz Suprasil cells thermostated at 20 °C. Absorption maxima $\lambda_{max}$ and molar absorption coefficients $\varepsilon_{max}$ are given in nm and l·mol$^{-1}$·cm$^{-1}$, respectively.

**Tris(pyrazol-2-yl)methane, tpm.[2]** To a vigorously stirred solution of pyrazole (14.94 g, 0.22 mol) and tetrabutylammonium bromide (3.54 g, 11 mmol) in water (220 ml) was slowly added sodium hydrogencarbonate (139.7 g, 1.66 mol). After cooling to room temperature chloroform (110 ml) was added and the mixture heated to reflux for 72 h. Then, excess base was removed by filtration. To the filtrate was added water (500 ml) followed by diethylether (300 ml). The organic phase was separated, washed with water (200 ml) as well as brine (200 ml) and then dried over anhydrous magnesiumsulfate. The solvent was removed *in vacuo* and the crude product recrystallized from cyclohexane to give a white crystalline solid. Yield 10.83 g, 50.6 mmol (69.3 %). Anal. Calcd (Obs) for $C_{10}H_{10}N_6$: C, 56.07 (55.94); H, 4.70 (4.62); N,

39.23 (39.07); IR (ATR): 614, 752, 799, 837, 916, 969, 1040, 1086, 1204, 1271, 1294, 1318, 1355, 1386, 1428, 1515, 2977, 3123; MS (FAB, 3-NBA matrix): *m*/*z* 147 $[M\text{-}C_3H_3N_2]^+$, 215 $[M+H]^+$, 237 $[M+Na]^+$; $^1$H NMR (200 MHz, $CDCl_3$): $\delta$ 8.43 (s, 1H, $pz_3CH$), 7.67 (d, 3H, $^3J$ = 1.4 Hz, $H_3$-pz), 7.58 (d, 3H, $^3J$ = 2.6 Hz, $H_5$-pz), 6.37 (dd, 3H, $J$ = 2.4 Hz, $J$ = 1.8 Hz, $H_4$-pz); $^{13}$C NMR (50 MHz, $CDCl_3$): $\delta$ 141.93, 129.64, 107.43, 83.36.

**$[Mn(CO)_3(tpm)]PF_6$, 1.**[3] A solution of manganese pentacarbonyl bromide (0.22 g, 0.8 mmol) and tris(pyrazol-2-yl)methane (tpm) (0.25 g, 1.2 mmol) in anhydrous acetone was heated to reflux under nitrogen protected from light for 5 h. The solvent was then removed *in vacuo*. The yellow residue was washed with diethylether and redissolved in 20 ml of methanol. Ammonium hexafluorophosphate (0.13 g, 0.8 mmol) was added to precipitate the product, which was filtered off, washed with water and diethylether and dried in vacuo. The resulting yellow crystalline powder was stored in the dark under nitrogen. If necessary, it could be crystallized by cooling a solution in methanol to -20 °C overnight. Yield 0.15 g, 0.3 mmol (38 %). Anal. Calcd (Obs) for $C_{13}H_{10}F_6Mn_1N_6O_3P_1$: C, 31.34 (30.75); H, 2.02 (1.80); N, 16.87 (17.23); IR (ATR): 828, 1061, 1097, 1260, 1293, 1411, 1452, 1664, 1941 (C=O), 2047 (C=O), 2961, 3144 cm$^{-1}$; MS ($ESI^+$, $CH_3OH$): *m*/*z* 353 $[M\text{-}PF_6]^+$, 851 $[2M+PF_6]^+$; $^1$H NMR (400 MHz, acetone-$d_6$): $\delta$ 9.65 (s, 1H, $C_\alpha$), 8.63 (d, 3H, $^3J$ = 2.0 Hz, $H_3$-pz), 8.57 (d, 3H, $^3J$ = 1.6 Hz, $H_5$-pz), 6.74 (m, 3H, $H_4$-pz); $^{13}$C NMR (100 MHz in acetone-$d_6$): 149.06, 136.44, 109.73, 76.19 (the carbonyl carbon atoms are in general very hard to detect in this class of compounds and could not be identified in this case).

## Myoglobin assay

In a quartz cuvette, horse skeletal muscle myoglobin (Fluka) dissolved in 0.1 M phosphate buffer (pH = 7.3) degassed by bubbling with nitrogen was reduced by addition of an excess of sodium dithionite in the same solvent and then buffer added to a total volume of 749 μl. To this solution, 1 μl of complex **1**, $[Mn(CO)_3(tpm)]PF_6$, dissolved in dimethylsulfoxide was added to give a final concentration of 20 μmol·l$^{-1}$ of manganese complex and 75 μmol·l$^{-1}$ of myoglobin with $A_{557\ nm} < 1$. For the study of the Soret band region, solutions were diluted ten-fold. Solutions were then either kept

in the dark or irradiated for given time intervals under nitrogen at 365 nm with a UV hand lamp (same as used in the cytotoxicity measurements) positioned perpendicular to the cuvette at a distance of 12 cm. Irradiations were interrupted in regular intervals to take UV/Vis spectra on a Varian Cary 100 spectrophotometer.

**Cell culture conditions**

The human HT-29 colon cancer cell line was obtained from the Institute of Pharmacy of the Free University in Berlin. The cell line was cultured in McCoy medium (Sigma, Germany) supplemented with 10 % fetal calf serum (Gibco, Germany), 2 mM *L*-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated in 75 $cm^2$ cell culture flasks at 37 °C in a humidified atmosphere of 5 % $CO_2$. The cell lines were passaged weekly using 0.05 % trypsin with 0.02 % EDTA (Gibco, Germany).

**Cytotoxicity assays**

The crystal violet assay[4] was performed with the HT-29 cell line in 96-well microtiter plates. 100 µl of a 40000 cells/ml suspension in culture medium were plated into each well and incubated for 24 h at 37 °C under 5 % $CO_2$. Then, 1 µl of a stock solution of the respective compound dissolved in dimethylsulfoxide/water (1:1) was added to 99 µl of fresh culture medium to reach the desired concentration of 0.5 % dimethylsulfoxide. 100 µM concentrations were tested in sixfold for compound **1**. Vehicle controls and 5-fluorouracil at 20 µM as a positive control were also applied. After application of the substances to the wells, the cells were kept in the dark for 24 h. Cells were then washed with 100 µl PBS buffer and afterwards covered with 100 µl of RPMI 1640 medium without phenolred (PAA Laboratories, Austria) and fetal calf serum. The plates were subsequently exposed for 10 min to UV light at 365 nm from a 6 W lamp (UVITEC, UK) positioned at a distance of 11.5 cm. Afterwards, cells were provided with another 100 µl of fresh culture medium and incubated for another 24 h in the dark. Cells were washed two times with colourless RPMI 1640 medium without phenol red and without fetal calf serum. Then, cell biomass was determined by the crystal violet assay. The medium was removed and cells were fixed with 4 % paraformaldehyde in PBS. Cells were then washed with PBS and afterwards with 0.1

% Triton-100 (Sigma) in PBS. Cells were then stained with a 0.04 % solution of crystal violet in 4 % ethanol and subsequently washed four times with ultrapure water (Millipore). Crystal violet was extracted with 96 % ethanol and the absorbance determined at 570 nm with a Tecan Saphire$^2$ microplate reader.

**Cellular Uptake Studies**

Cellular uptake studies were performed according to a previously described method with minor modifications.[5] In short, HT-29 cells were grown until at least 70 % confluency in 175 cm$^2$ cell culture flasks. Stock solutions of **1** in dimethylsulfoxide (DMSO) were freshly prepared and diluted with cell culture medium to the desired concentrations (final DMSO concentration: 0.1 % v/v, final concentrations of **1**: 10 - 100 µM). The cell culture medium of the cell culture flasks was replaced with 10 mL of the cell culture medium solutions containing compound **1** and the flasks were incubated at 37°C / 5 % $CO_2$ for 6 h.

The cell pellets were isolated, resuspended in 1 mL doubly-destilled water, lysed with a sonotrode and appropriately diluted using doubly-destilled water. An aliquot was removed for protein quantification by the Bradford method. Prior to manganese quantification by GF-AAS (see below) to each 200 µL of the cell suspensions 20 µL Triton X-100 (1 %) and nitric acid (13 %) were added. Cellular uptake is expressed as nmol manganese per mg cell protein (Figure S1). The manganese background concentration of blank pellets was subtracted from the probes. Data were obtained from 2 - 3 independent experiments.

**Graphite Furnace Atomic Absorption Spectroscopy (GF-AAS)**

A Vario-6 graphite furnace atomic absorption spectrometer (AnalytikJena AG) was used for the manganese determination. Manganese was detected at a wavelength of 279.5 nm with a bandpass of 0.2 nm. A deuterium lamp was used for background correction. Probes were injected at a volume of 20 µL into graphite tubes. Heating procedures in the graphite furnace are given in Table S1. The graphite tube was purged with a constant flow of argon gas which was only halted during the zeroing and atomisation steps. The mean AUC (area under curve) absorptions of duplicate

injections were used throughout the study. Serial aqueous dilutions of **1** were used for calibration purposes.

Table S1. Graphite furnace program.

| step | T (°C) | rate (°C·s$^{-1}$) | hold (s) |
|---|---|---|---|
| drying | 90 | 10 | 40 |
| drying | 105 | 7 | 30 |
| drying | 120 | 15 | 20 |
| drying | 500 | 50 | 30 |
| pyrolysis | 1000 | 200 | 20 |
| AZ (zeroing) | 1000 | 0 | 5 |
| atomisation | 1600 | 1000 | 4 |
| tube cleaning | 2200 | 1000 | 6 |

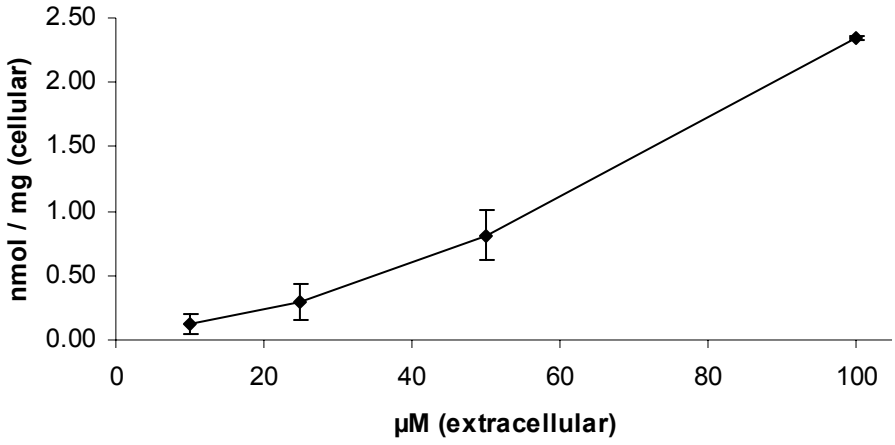


Figure S1: Cellular uptake of **1** into HT-29 cells (n = 2 - 3).

**X-ray crystallographic data collection and refinement of the structure of 1.**

A yellow single crystal of **1** (0.30 x 0.30 x 0.10 mm) was coated with perfluoropolyether, picked up with a glass fiber, and immediately mounted in the nitrogen cold stream of the diffractometer. Intensity data were collected at 213(2) K using graphite monochromated Mo-K$\alpha$ radiation ($\lambda$ = 0.71073 Å). Final cell constants were obtained from a least squares fit of a subset of 9772 strong reflections. Data collection was performed by hemisphere runs taking frames at 0.3° in $\omega$ on a Bruker AXS SMART CCD diffractometer. The program SADABS[6] was used to account for

absorption. The SHELXL-97 software package[7] was used for solution, refinement, and artwork of the structure. The structure was readily solved by Patterson methods and difference Fourier techniques. All non-hydrogen atoms were refined anisotropically and hydrogen atoms were placed at calculated positions and refined as riding atoms with isotropic displacement parameters. See Tables S2, S3 and S4 for more details on the structure refinement, atomic coordinates, and bond lengths and angles. Crystallographic data (excluding structure factors) has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no CCDC 670201 for structure **1**. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK [Fax: int. code +44(1223)336-033; E-mail: deposit@ccdc.cam.ac.uk

## References

[1] H. E. Gottlieb, V. Kotlyar, A. Nudelman, *J. Org. Chem.* **1997**, *62*, 7512-7515.
[2] D. L. Reger, T. C. Grattan, K. J. Brown, C. A. Little, J. J. S. Lamba, A. L. Rheingold, R. D. Sommer, *J. Organomet. Chem.* **2000**, *607*, 120-128.
[3] S. Trofimenko, *J. Am. Chem. Soc.* **1970**, *92*, 5118-5126.
[4] G. Bernhardt, H. Reile, H. Brinböck, T. Spruß, H. Schönenberger, *J. Cancer. Res. Clin. Oncol.* **1992**, *118*, 35-43.
[5] I. Ott, H. Scheffler, R. Gust, *ChemMedChem* **2007**, *2*, 702-707.
[6] G. M. Sheldrick, *SADABS, Program for Empirical Correction of Area Detector Data*, Universität Göttingen, Germany, **1996**.
[7] G. M. Sheldrick, *SHELXL-97, Program for Crystal Structure Refinement*, Universität Göttingen, Germany, **1997**.