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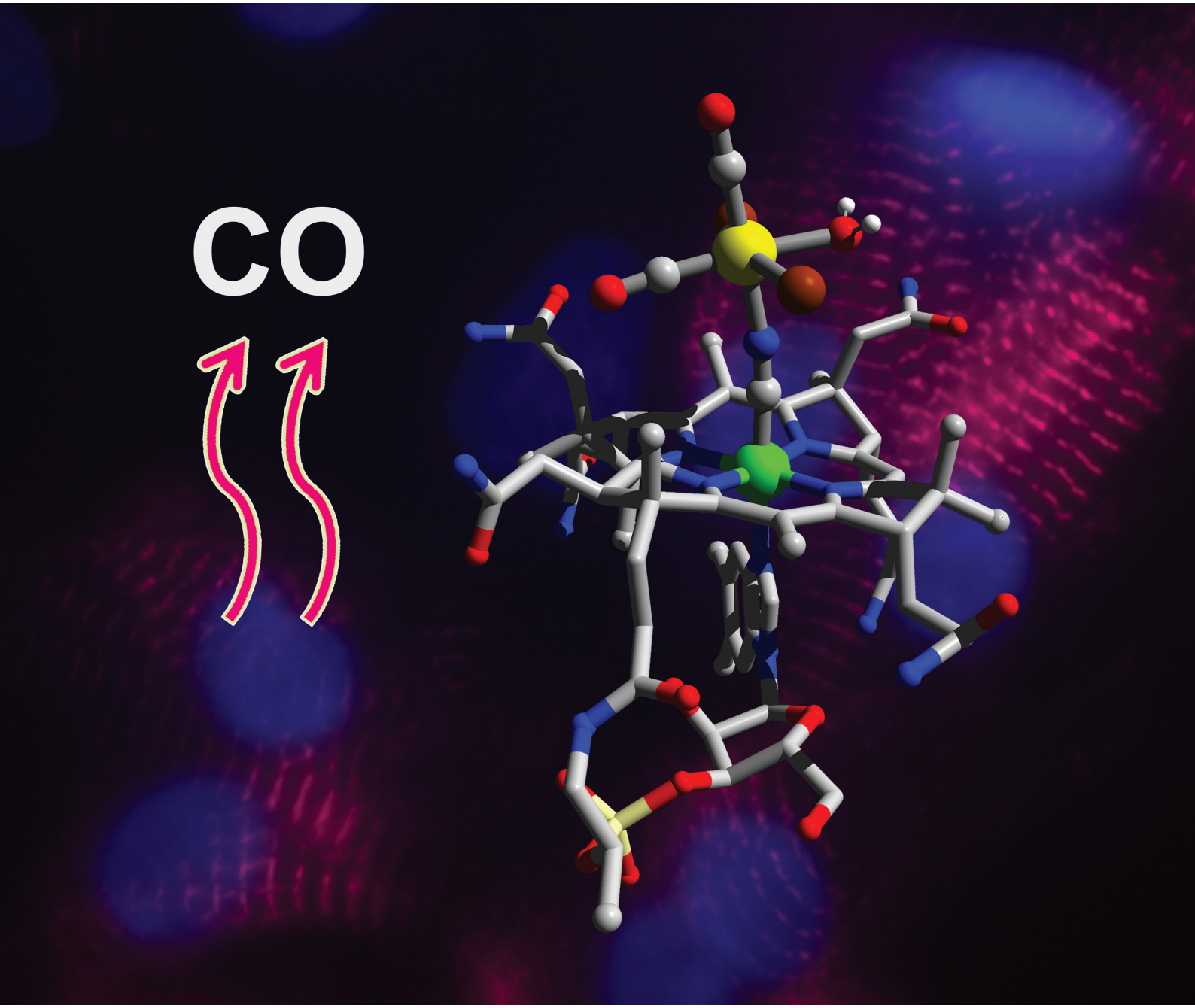

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PAPER

17 e⁻ rhenium dicarbonyl CO-releasing molecules on a cobalamin scaffold for biological application†

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Cyanocobalamin (B_{12}) offers a biocompatible scaffold for CO-releasing 17-electron dicarbonyl complexes based on the *cis-trans*-$[Re^{II}(CO)_2Br_2]^0$ core. A Co–C≡N–Re conjugate is produced in a short time and high yield from the reaction of $[Et_4N]_2[Re^{II}Br_4(CO)_2]$ (ReCORM-**1**) with B_{12}. The B_{12}-$Re^{II}(CO)_2$ derivatives show a number of features which make them pharmaceutically acceptable CO-releasing molecules (CORMs). These cobalamin conjugates are characterized by an improved stability in aqueous aerobic media over the metal complex alone, and afford effective therapeutic protection against ischemia-reperfusion injury in cultured cardiomyocytes. The non-toxicity (at μM concentrations) of the resulting metal fragment after CO release is attributed to the oxidation of the metal and formation in solution of the ReO_4^- anion, which is among the least toxic of all of the rare inorganic compounds. Theoretical and experimental studies aimed at elucidating the aqueous chemistry of ReCORM-**1** are also described.

Introduction

In the last decade, the use of carbon monoxide (CO) as a cytoprotective and homeostatic molecule has received increasing attention in medicine due to its documented beneficial therapeutic effects.[1,2] There are three main areas where CO is evaluated as a clinically valuable medical agent: 1) inflammation, 2) cardiovascular diseases and 3) organ preservation and transplantation. The anti-inflammatory properties of CO have been corroborated in a large number of animal models including cerebral malaria inflammation,[3] rheumatoid arthritis,[4] autoimmune neuroinflammation,[5] sickle cell disease,[6,7] diabetes[8,9] and acute hepatitis.[10,11] The protective effects of CO as a vasodilator have been successfully evaluated for several cardiovascular diseases including pulmonary arterial hypertension, for which there is no cure.[12] Carbon monoxide proved effective in prolonging organ graft survival, particularly in heart and kidney transplants for which CO inhalation (3 mg per kg for 1 h) has entered Phase II clinical trials.[2]

Despite its current evaluation in novel therapies, the use of gaseous CO poses several problems related to safe handling and delivery to specific target sites in a controlled and measurable fashion. The challenges associated with clinical application of the gas by inhalation have sparked the design of CO-releasing molecules (CORMs) as an alternative approach to the administration of CO (*e.g.* orally or by injection). Transition metal carbonyl complexes have been predominantly evaluated as CORMs. There are numerous examples available in the literature,[13–25] of which three molecules have been most extensively investigated in biology and medicine: the lipid-soluble tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$, CO-RM2),[26–28] the water soluble tricarbonylglycinatoruthenium(II) monomer ($[Ru(CO)_3Cl(glycinato)]$, CO-RM3)[29–34] and the boron-containing carboxylic acid $Na_2[H_3BCO_2]$ salt (CO-RMA1).[35–38] Treatment of several diseases with these CORMs has shown that the molecules elicit the same type of therapeutic effects as CO gas.[2]

Design, identification and characterization of new CORMs constitutes an active field of research not only because of the aforementioned problems associated with the clinical application of the gas, but also because in some cases the use of CORMs has an advantage over CO inhalation. For instance, the bactericidal effect of CO-RM3 can prevent sepsis-induced death in mice, while CO gas does not seem to directly affect bacterial survival.[32] As recently pointed out,[2] none of the characterized CO-releasing carbonyl complexes displays acceptable properties as required for a medical drug. Motterlini and Otterbein put forward a number of requirements CORMs should meet.[2] These include: 1) water solubility and biocompatibility, 2) stability in aqueous aerobic media, 3) slow decay of the M(CO)*x* fragment in the blood, 4) low toxicity and rapid excretion of the metal scaffold after CO release.

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† Electronic supplementary information (ESI) available: Spectroscopic details (HPLC, MS, IR, UV-Vis, ^{1}H-NMR) of B_{12}-ReCORM-**2** and **4** and of molecules **3** and **5**. Theoretical details and UV-Vis absorption spectra. Effect of CORMs' supplementation on the oxygen levels in the cell-free culture medium and on NRCs respiration. CCDC reference numbers 817626 and 817627 (compounds **3** and **5**). For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c1dt10649j

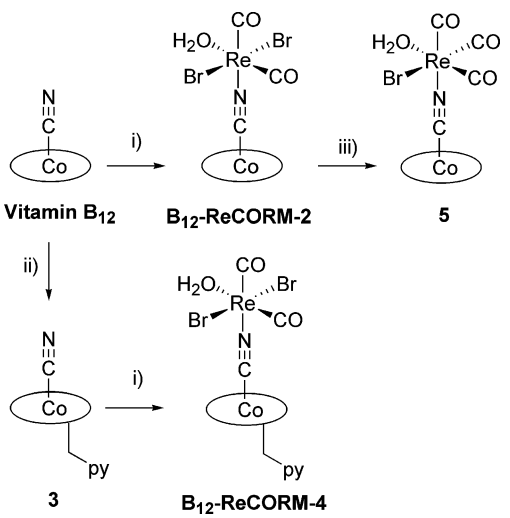
Within this context, we have recently described a family of Re^{II}-based CORMs.[39,40] These uncommon monomeric 17-electron dicarbonyl species are unique in the field as the rate of CO-release (and thus the decay of the $Re(CO)_2$ fragment) can be conveniently modulated by pH and by ligand variation on the basic *cis-trans*-$[Re(CO)_2Br_2]^0$ core. The molecules were further shown to protect cultured cardiomyocytes against ischemia-reperfusion injury by increasing cell survival by up to 83%.[39] However, most complexes showed poor water solubility. In order to meet the above mentioned requirement for a pharmaceutically acceptable CORM, we have begun to study the reactivity of the $[Et_4N]_2[Re^{II}Br_4(CO)_2]$ (ReCORM-**1**) salt with biomolecules. In this contribution we present cyanocobalamin (B_{12}) derivatives of 17-electron rhenium dicarbonyl species which, as a second generation of Re^{II}-based CORMs, show several improved features over the original complexes. The derivatives herein described: *a*) are fully water soluble and biocompatible, *b*) show improved stability in aqueous aerobic media over the metal complex alone, *c*) are non-toxic towards cultured cardiomyocytes, *d*) protect these cells against ischemia-reperfusion injury and *e*) after CO release, in water under aerobic conditions, the rhenium complex is oxidized to ReO_4^- which is among the least toxic of all of the rare inorganic compounds.[41]

Finally, as it is our intention to further develop these potential drugs, we have devised for the species a general nomenclature of the type: X-MCORM-#; where X = carrier biomolecule (here B_{12}); M = transition metal (here Re); CORM explicitly denotes the function; and # = associated numeric value to differentiate the molecules. This nomenclature is used throughout the text.

Results and discussion

Synthesis and characterization

The B_{12}-$Re^{II}(CO)_2$ adduct (B_{12}-ReCORM-**2**, see Scheme 1) was prepared in good yield from the reaction of cyanocobalamin and the $[Et_4N]_2[Re^{II}Br_4(CO)_2]$ (ReCORM-**1**) salt in methanol. HPLC-MS traces recorded during the reaction revealed the formation of a single product in quantitative yield with a mass of $m/z = 1758.4$ and an isotope pattern corresponding exactly to the theoretical mass of B_{12}-ReCORM-**2** after loss of a solvent molecule (see ESI†). The absence of a negative MS signal corresponding to an adduct with three coordinated bromides and the recovery of nearly two equivalents of $[Et_4N]Br$ after workup are all in agreement with the proposed formulation of B_{12}-ReCORM-**2**. The IR spectrum of B_{12}-ReCORM-**2** showed a single ν_{CN} band at 2184 cm^{-1}, which is shifted by 50 cm^{-1} to higher frequency than the corresponding stretching vibration of B_{12}. Similar shifts to higher energy have been reported for other B_{12}-metal complex adducts and are characteristic for bridging cyanides.[42–44] Alongside the ν_{CN} band at 2184 cm^{-1} the IR spectrum of B_{12}-ReCORM-**2** shows two ν_{CO} stretching frequencies at 1989 and 1839 cm^{-1} which are characteristic of *cis-trans*-$[Re^{II}(CO)_2Br_2L_2]$ complexes (ESI†).[39,40]



Scheme 1 Synthesis of B_{12}-adduct **3** and B_{12}-$Re^{II}(CO)_2$ conjugates B_{12}-ReCORM-**2** and B_{12}-ReCORM-**4**. Reagents and conditions: i) $[Et_4N]_2[Re^{II}Br_4(CO)_2]$ (ReCORM-**1**), methanol/water, 1.5 h, 40–50 °C; ii) CDI, DMSO, 12 h, 60 °C followed by 4-picolylamine, 0.5 h, RT; iii) ReCORM-**1**, methanol/water, 12 h, 40–50 °C.

The anion of ReCORM-**1** rapidly coordinates to the cyano group of B_{12}. Under our conditions the reaction is complete in *ca.* 90 min. In comparison similar adducts of cyanocobalamin with *fac*-$[Re^{I}(CO)_3]^+$ complexes and cisplatin are formed at higher temperatures after prolonged reaction times (up to 16 h).[42–44] If the reaction is allowed to proceed for 12 h the major B_{12}-Re adduct was identified as a B_{12} complex of *fac*-$[Re^{I}(CO)_3Br(H_2O)]^+$. This product (**5** in Scheme 1) was isolated and it was possible to grow X-ray quality crystals (Fig. 1).[45] Compound **5** crystallized in the $P2_12_12_1$ space group. The Re atom (*ca.* 50% occupancy) sits on top of the cyano ligand with a Re–N distance of 2.24 Å, *ca.* 0.09 Å longer than the same distance found in the other two known B_{12}-$Re^{I}(CO)_3$ adducts.[42] The C–N–Re angle has a value of 160° being significantly more acute than those found in Re and Pt analogues.[42–44] The cobalamin bond lengths and angles are similar to those of native B_{12}. Due to the inherent instability of B_{12}-ReCORM-**2** (*vide infra*) it was not possible to obtain single X-ray quality crystals of the compound. However, in order to obtain insights into the structure of this adduct, calculations at the density functional level of theory were performed. The calculated structure of B_{12}-ReCORM-**2** is shown in Fig. 1. Bond lengths and angles around the Re coordination sphere were found to be in good agreement with those of the *cis*-$[Re^{II}(CO)_2Br_3(NCCH_3)]^-$ anion previously published.[40] The DFT calculations also correctly predicted the bending of the two coordinated *trans* bromides away from the CO ligands always observed in the solid state structures of the family of *cis-trans*-$[Re^{II}(CO)_2Br_2L_2]$ complexes.

The UV-visible spectrum of B_{12}-ReCORM-**2** is virtually identical to that of native B_{12} except for a pronounced absorption at 406 nm ascribed to the presence of the coordinated Re^{II} complex (Fig. 2).[39,40] In water the intensity of this signal decreased over time. Theoretical calculations of a model *cis-trans*-$[Re^{II}(CO)_2Br_2(H_2O)(N{\equiv}C{-}H)]$ complex revealed that in water the observed changes are consistent with the substitution of the bound *trans* bromide ions by water molecules and the formation of a *cis*-$[Re^{II}(CO)_2(H_2O)_3(N{\equiv}C{-}H)]^{2+}$ species (ESI†). The presence in solution of the B_{12}-*cis*-$[Re^{II}(CO)_2(H_2O)_3]^{2+}$ adduct (**2a** in Scheme 2, *vide infra*) was also confirmed *via* HPLC-MS analysis.

In order to assess the versatility of B_{12}-ReCORM-**2** for applications in biology, knowledge about the water stability of the compound is crucial. Immediately after dissolution in water (at 25 °C, aerobic conditions), the HPLC-MS trace reveals the

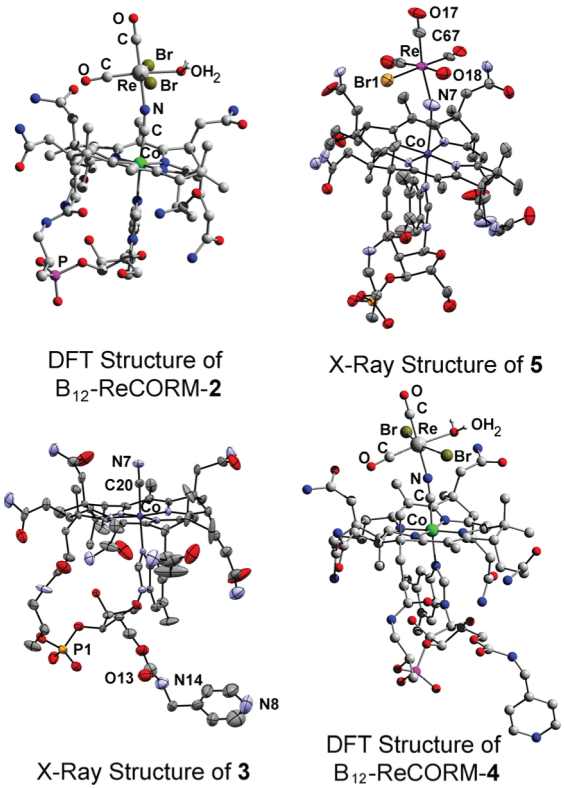


Fig. 1 DFT calculated structures of B_{12}-ReCORM-**2** and B_{12}-ReCORM-**4** and ORTEP plots of X-ray structures of **3** and **5**. Ellipsoids are drawn at 50% probability, hydrogen atoms are omitted for clarity. Selected bond lengths (Å) and angles (°) for **5** are: Re–Br1 2.6482(19); Re–O18 2.072(12); Re–N7 2.221(7); Re–C67 1.926(16); Br1–Re–N7 89.32(18), O18–Re–N7 79.3(4); C67–Re–N7 170.5(6). Crystallographic details are summarized in the ESI.†

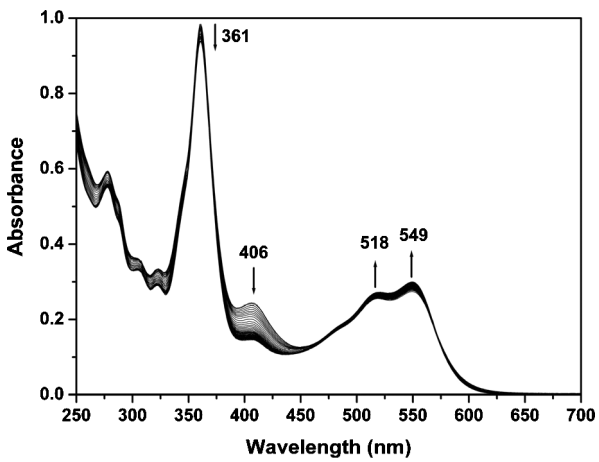
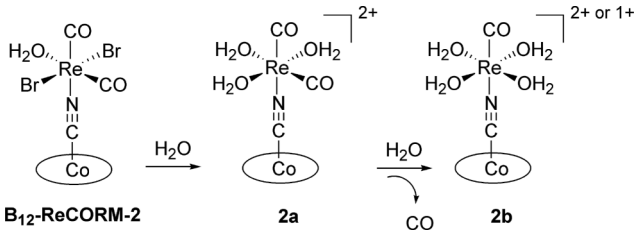


Fig. 2 Changes in the UV-visible spectrum of an unbuffered aqueous solution of B_{12}-ReCORM-**2**. Spectra were recorded at fixed intervals of 5 min at 25 °C.

presence of a single peak ascribed to B_{12}-ReCORM-**2**. After 30 min six distinct peaks were observed. These were identified by HPLC-MS as a mixture of B_{12}-ReCORM-**2**, **2a**, **2b** free B_{12}, aquocobalamin, ReO_4^- and a Re complex whose coordination sphere could not be unambiguously assigned (Scheme 2, ESI†). It is clear that after dissolution in water the $Re^{II}(CO)_2$ species in



Scheme 2 The postulated sequence of events leading to CO-release from B_{12}-ReCORM-**2**.

B_{12}-ReCORM-**2** exchanged the coordinated bromides for water molecules. It is however difficult to pinpoint the right sequence of events which lead to the formation of a mono carbonyl species after release of carbon monoxide from the *cis*-$[Re^{II}(CO)_2]$ core. Analysis of the kinetics of CO release is described in the following paragraphs. Here it should be noted that solvation of B_{12}-CORM-**2** and CO release might be concurrent events. However, the absence of MS signals corresponding to a Re mono carbonyl species containing bromide ions is in support of the sequence illustrated in Scheme 2.

We have previously shown that the $[Et_4N]_2[Re^{II}Br_4(CO)_2]$ salt (ReCORM-**1**) reacts with pyridine and imidazole type ligands yielding stable complexes.[39,40] In order to further improve the stablility of the B_{12}-$Re^{II}(CO)_2$ adduct, B_{12} was derivatized with a pendent pyridine arm at the 5′-OH position of the ribose according to a modified procedure recently described by Grissom *et al.* (**3** in Scheme 1).[46] The X-ray structure of **3** is shown in Fig. 1 confirming the presence of a pyridine unit on the sugar moiety.[47] The adduct crystallized in the $P2_12_12_1$ space group. Bond lengths and angles are not significantly different than those of native B_{12}.

The reaction of **3** with ReCORM-**1**, under similar conditions to those described for the formation of B_{12}-ReCORM-**2**, gave a single product in quantitative yield (B_{12}-ReCORM-**4** in Scheme 1). The isolated product showed a mass of m/z = 1893.4 with an isotope pattern corresponding to the theoretical mass of B_{12}-ReCORM-**4** after loss of a solvent molecule. The IR spectrum of B_{12}-ReCORM-**4** showed a single ν_{CN} band at 2184 cm^{-1} and ν_{CO} stretching frequencies at 1989 and 1839 cm^{-1} at the same energy to those of B_{12}-ReCORM-**2**, indicating the formation of the same Co–C≡N–Re conjugate. Under conditions of limiting amounts of **3** (*i.e.* with 5 fold excess of ReCORM-**1**) we found evidence of a bis-Re adduct of **3** but by allowing the reaction to proceed for several hours, a mixture of products comprising derivatives of the *fac*-$[Re^{I}(CO)_3]^+$ core was obtained (ESI†). In water B_{12}-ReCORM-**4** showed similar behaviour to that described for B_{12}-ReCORM-**2**. The optimized structure of B_{12}-ReCORM-**4** is shown in Fig. 1. The calculated structure parameters are similar to those found in B_{12}-ReCORM-**2**.

CO releasing properties of B_{12}-ReCORM-2 and B_{12}-ReCORM-4

The carbon monoxide releasing properties of B_{12}-ReCORM-**2** and B_{12}-ReCORM-**4** were evaluated by the myoglobin assay (Fig. 3).[30,31] In these experiments, an aliquot of a freshly prepared concentrated solution of the cobalamin adducts was added to a buffered solution of horse skeletal myoglobin (Mb), freshly reduced with excess sodium dithionite under N_2. The conversion of Mb to carbon monoxide myoglobin (MbCO) was followed over

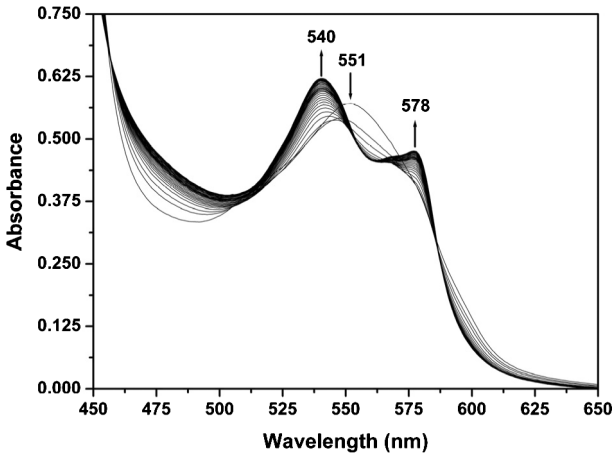


Fig. 3 A typical spectrum of conversion of deoxy-myoglobin (Mb) to carbonmonoxy myoglobin (MbCO) by cobalamin derivatives B_{12}-ReCORM-**2** and B_{12}-ReCORM-**4**. Here reproduced are the changes of the spectrum of a 30 μM Mb solution after the addition of B_{12}-ReCORM-**2** (30 μM, 25 °C, 0.1 M phosphate buffer pH 7.4, spectra intervals = 5 min).

time by measuring the changes in the absorption spectra of the Q band region of this protein at 25 °C and at pH 7.4 after addition of B_{12}-ReCORM-**2** and B_{12}-ReCORM-**4**. The maximum absorption peak of Mb at 551 nm was rapidly converted over time to the spectrum of MbCO, with two maximum absorption peaks at 540 and 578, respectively. A typical spectrum is shown in Fig. 3.

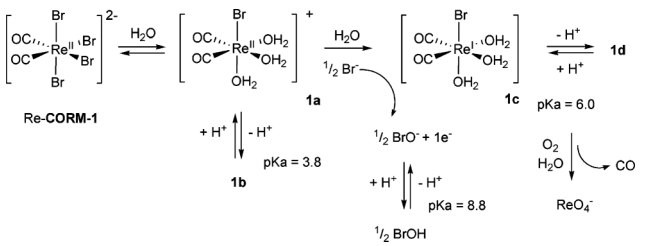
Both compounds elicited the spectral changes associated with CO release. The amount of MbCO formed over time after addition of the cobalamin adducts to the Mb solution was calculated according to the known extinction coefficients.[30,31] The rate of CO loss from B_{12}-ReCORM-**2** and B_{12}-ReCORM-**4** was found to be the same within experimental error with a half life ($t_{1/2}$) of *ca.* 20 min. In comparison ReCORM-**1** under the same conditions shows a $t_{1/2}$ for CO release of 6 min.[39] This difference further corroborates the notion that simple ligand substitution of the *cis*-*trans*-$[Re^{II}(CO)_2Br_2]^0$ core is able to modulate the kinetics of the thermal dissociation of carbon monoxide and thus the decay of the $Re(CO)_2$ fragment. When the experiments were performed under conditions of a limiting amount of B_{12}-ReCORM-**2** or B_{12}-ReCORM-**4**, taking into account the molar extinction coefficient of MbCO, it was found that approximately 1 mol of CO was released per mole of the corresponding complex within 2 h.

Aqueous chemistry of ReCORM-1

The aqueous chemistry of ReCORM-**1** was studied in detail in order to delineate a possible mechanism of CO release from the compounds herein described. Due to the rapid CO loss, the paramagnetic nature of ReCORM-**1** and the lack of ligands bearing active NMR probes the main techniques employed in this study were IR spectroscopy and MS. The salt of ReCORM-**1** is soluble in water and shows two absorption maxima at 335 and 389 nm (ESI†). When a 1 mM solution of ReCORM-**1** was prepared in distilled water the pH dropped rapidly to 3. The liquid IR spectrum of ReCORM-**1** in phosphate buffer (0.1 M, pH = 7.4) showed three distinct signals within only a few minutes after dissolution. Two signals were observed at 1884 and 1782 cm^{-1} together with a small peak at 1925 cm^{-1} (ESI†). These signals slowly disappeared and after *ca.* 30 min a single major peak was detected at a frequency of 1887 cm^{-1} (ESI†).

The liquid IR evidence in phosphate buffer clearly points to a reduction of the *cis*-$[Re^{II}(CO)_2]^{2+}$ core and formation of a complex comprising a *cis*-$[Re^{I}(CO)_2]^{+}$ moiety. This reduction is evidenced by the shift of carbonyl stretching frequencies of *ca.* 100 cm^{-1} to lower frequency when compared to the spectrum of ReCORM-**1** in methanol (ν_{CO} frequencies at 2000 and 1856 cm^{-1}, see reference 40). After 30 min the presence of a single C≡O stretching frequency indicates a monocarbonyl complex, pointing at the release of 1 equivalent of CO. The reduction of ReCORM-**1** in water was further confirmed by MS data (ESI†). The MS spectrum of an aqueous solution of ReCORM-**1**, recorded soon after sample preparation (ESI†), showed two clear peaks assigned to a $[Re^{II}(CO)_2Br_3]^-$ ion (m/z = 481.7) and to a reduced $[Re^{I}(CO)_2Br_2(H_2O)(CH_3OH)]^-$ species (m/z = 454.0). After 12 h the same sample showed signals at m/z = 250.2 and 428.9 assigned to ReO_4^- and the monocarbonyl $[Re^{I}(CO)Br_2(H_2O)_3]^-$ complex respectively (ESI†).

Rapid titration of an aqueous solution of ReCORM-**1** (0.1 M KNO_3, 25 °C, completed within 15 min) indicated Br^- as the possible reducing agent. Base titration of an aqueous solution of ReCORM-**1** showed three distinct acid–base equilibria (ESI†). The corresponding pK_a values are 3.8, 6.0 and 8.9. The first two steps are tentatively assigned to the formation of the mononuclear deprotonation products *cis*-$[Re^{II}(CO)_2Br(H_2O)_2(OH)]$ and *cis*-$[Re^{I}(CO)_2Br(H_2O)_2(OH)]^-$ (**1b** and **1d** respectively in Scheme 3). The pK_a value at 8.9, on the other hand, is attributed to the presence in solution of hypobromite (BrO^-), in agreement with the value reported for the oxidation of bromide-containing water and hypobromite formation.[48]



Scheme 3 A possible mechanism underlying the CO-release from ReCORM-**1** and its subsequent oxidation to the ReO_4^- anion.

Taken together, these results suggest the following mechanism for the CO release of ReCORM-**1** (Scheme 3). When dissolved in water three bromides in ReCORM-**1** are rapidly exchanged for solvent molecules. Theoretical calculations of the progressive aquation of ReCORM-**1** are in full support of this assignment (ESI†). Of the calculated UV-Vis spectra of the different aquo complexes the only one closely matching the experimental spectrum (two absorption maxima at 335 and 389 nm) is the one assigned to species **1a** in Scheme 3 (ESI†). Substitution of three Br^- by three water molecules is postulated to increase the $Re^{I} \rightarrow Re^{II}$ redox couple of **1a** to a value sufficient to drive the oxidation of Br^- to BrO^-. The value for the $Re^{I} \rightarrow Re^{II}$ redox couple of ReCORM-**1** is reported at *ca.* +0.1V (*vs.* NHE).[40] According to the electrochemical parameters described by Lever,[49] substitution of three Br^- by three water molecules should increase a metal M(*n*–*1*)/M(*n*) redox potential by +0.78 V. This value is

already sufficient to drive the oxidation of bromide in water to hypobromite whose value for the redox reaction is reported as +0.76 V (*vs.* NHE).[48]

Following reduction of the solvated Re^{II} complex the corresponding Re^{I} species is formed (**1c** in Scheme 3) which may undergo further acid–base equilibrium (pK_a 6.0) to generate the *cis*-$[Re^{I}(CO)_2Br(H_2O)_2(OH)]^-$ complex (**1d**). One of the reduced *cis*-$[Re^{I}(CO)_2]$ species is then postulated to be the CO-releasing entity liberating 1 equivalent of carbon monoxide within 30 min under physiological conditions. Finally in water under aerobic conditions, the monocarbonyl Re^{I} complex (clearly detected by liquid IR and MS, ESI†) is oxidized to ReO_4^-.

Cytoprotective effects of B_{12}-ReCORMs

B_{12}-ReCORM-**2** and **4** were tested for their cytotoxic and their cytoprotective effects using the neonatal rat cardiomyocyte (NRC) cell-based model of ischemia-reperfusion injury (I/R) as previously described.[39] Cytotoxicity of some Re^{II}-based CORMs observed earlier was most likely associated with their accumulation in the cells, followed by formation of cytotoxic intermediates such as 4-methylpyridine which may interfere with the cell metabolism.[39] The membrane-impermeable Re^{II}-CORMs studied so far were non-toxic in the micromolar concentration range. To test for the possible uptake of B_{12}-ReCORMs by cells, incubation of NRCs with 30 μM of B_{12}-ReCORM-**2** and **4** was performed and cell culture medium samples were collected over 180 min of incubation. Atomic absorption spectroscopy (AAS) measurements showed that the rhenium concentration in the medium supplemented with B_{12}-ReCORM-**2** and B_{12}-ReCORM-**4** did not change over time. This observation implies that complexes (or the dissociated Re fragment) did not enter the cells through the cell surface membrane during a 3 h incubation period (data not shown). The fraction of dead cells tended to decrease in the presence of CORMs, but, due to the high variability, the differences were not statistically significant (2.7 ± 1.7% in control *vs.* 1.1 ± 0.3 and 1.0 ± 0.2 in the presence of B_{12}-ReCORM-**2** and B_{12}-ReCORM-**4** respectively).

Exposure of the NRCs to the conditions mimicking ischemia-reperfusion (I/R) resulted in a 5-fold increase in the number of dead cells (control in Fig. 4). Administration of 30 μM B_{12}-ReCORM-**2** at the "onset of reperfusion" nearly prevented cell mortality (cell death was reduce by *ca.* 80% as compared to control) whereas 30 μM B_{12}-ReCORM-**4** reduced cell death by *ca.* 50%. Thus, B_{12}-ReCORM-**2** proved to be more efficient in preventing I/R-induced cell death than B_{12}-ReCORM-**4**. Therefore B_{12}-ReCORM-**2** was chosen for further investigation and compared to $[Et_4N]_2[Re^{II}Br_4(CO)_2]$ (ReCORM-**1**) and the previously reported *cis*-*trans*-$[Re^{II}(CO)_2Br_2(Im)_2]$ complex (where Im = imidazole, ReCORM-**6**) that also exhibited substantial cytoprotective effects.[39] All three compounds release CO in aqueous solution, albeit at different rates, and with the exception of the lipophilic ReCORM-**6** show good water solubility. Under normal physiological conditions the compounds showed no cytotoxicity towards NCRs up to a tested concentration of 120 μM.

Carbon monoxide is known to suppress respiration in different cell types by inhibiting the mitochondrial electron transfer chain and by interfering with oxygen binding.[50] Therefore, the effect of the three selected CORMs on oxygen consumption by NRCs was investigated. None of the tested compounds displayed any

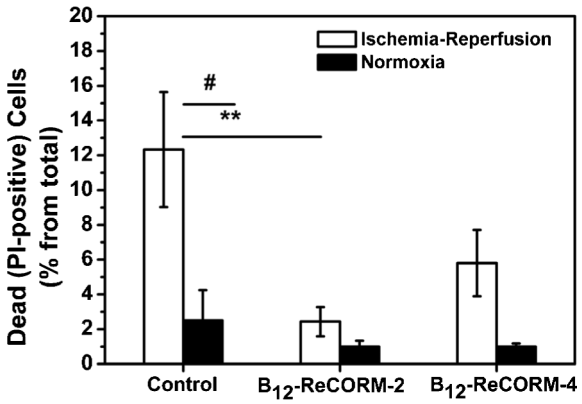


Fig. 4 Cytoprotective effects of conjugates B_{12}-ReCORM-**2** and B_{12}CO-RM-**4** (30 μM) against "ischemia-reperfusion" injury (I/R). Cell damage in neonatal rat ventricular cardiomyocytes (NRCs) after 16 h of ischemia (hypoxia, aglycemia, and acidosis). CORMs or the aliquot of solvent (DMSO) were added to the cell culture medium at the onset of the reperfusion period of 9 h at 30 μM concentration. Bars indicate the % of PI-positive (dead) cells. ** denotes $p < 0.01$ when I/R B_{12}-ReCORM-**2**-treated cells are compared to I/R control. # denotes $p < 0.05$ when normoxic cells are compared with I/R control.

significant effect on oxygen consumption by NRCs within the concentration range relevant for cytoprotection within 30 min of observation (ESI†). The lack of effect neither depended on the rate of CO release nor on the compound solubility. Interestingly, addition of ReCORM-**1**, and to a lesser extent of B_{12}-ReCORM-**2**, resulted in deoxygenation of the cell-free medium within minutes after administration. This CORM-induced deoxygenation was dose-dependent (ESI†) and most likely reflected the reaction describing the formation of ReO_4^- in Scheme 3. The deoxygenation efficiency followed the kinetics of CO release (and thus the decay of the $Re(CO)_2$ fragment) and was maximal for the fast-releasing ReCORM-**1** compound (Fig. 5A and ESI†).

Oxidative stress triggered by acute hyperoxygenation is a hallmark of reperfusion injury. Based on the findings presented in Fig. 5A, selected Re^{II}-based-CORMs may be viewed as oxygen scavengers which, when applied early on during reperfusion, may reduce the cellular oxidative damage. To test this hypothesis intracellular reduced glutathione (GSH) levels were determined in cells 10 min after the "onset of reperfusion" in the presence or absence of 30 μM ReCORM-**1**, ReCORM-**6**, and B_{12}-ReCORM-**2**. As shown in Fig. 5B, presence of the ReCORM-**1** complex at reperfusion resulted in an increase in the intracellular reduced glutathione (GSH) levels reflecting its antioxidative action. This acute antioxidative effect of ReCORM-**1** most likely reflects the ability of the intermediates formed upon CO release to react with oxygen ultimately yielding ReO_4^- (see Scheme 3).

Although the molecular mechanisms of the observed cytoprotective effects of the above-mentioned Re^{II}-based-CORMs remain unknown, their cytoprotective action may, at least in part, be attributed to the extracellular release of CO and to the de-oxygenating effect described above. Finally, our observations suggest that the protective effect of the Re^{II}-based CORMs is not related to a decrease in mitochondrial respiration and secondary free radical production.[50]

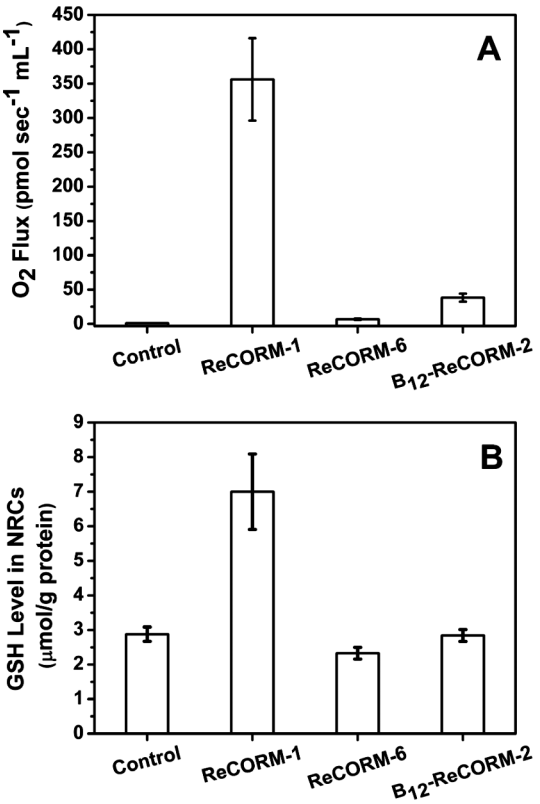


Fig. 5 Regulation of oxygen availability and the intracellular reduced glutathione (GSH) content by Re^{II}-based-CORMs. **A**: Reduction in oxygen content in the incubation medium within 10 min after addition of 60 μM of ReCORM-**1**, ReCORM-**6**, and B_{12}-ReCORM-**2**; N = 6–8. Given are means ± SEM. **B**: Intracellular reduced glutathione (GSH) content in NRCs exposed to 16 h of ischemia and 10 min of reperfusion in the presence or absence of Re^{II}-based-CORMs. N = 4. Data are means ± SEM.

Conclusions

Cyanocobalamin (B_{12}) derivatives of CO-releasing 17-electron rhenium dicarbonyl species were presented. As a second generation of Re^{II}-based CORMs, the B_{12} adducts were found to be fully water soluble, biocompatible and showed an improved stability in aqueous aerobic media over the original family of complexes alone. In all cases formation of a Co–C≡N–Re conjugate was observed from the reaction of the vitamin derivatives and the $[Et_4N]_2[Re^{II}Br_4(CO)_2]$ salt (ReCORM-**1**). Theoretical and experimental studies suggested that aquation of the bound bromide ions of the Re^{II}-based CORMs is the first step in the mechanism underlying CO release.

The B_{12}-$Re^{II}(CO)_2$ derivatives are non-toxic towards cultured cardiomyocytes and provided an effective therapeutic protection against ischemia-reperfusion injury to the cells. Experimental data suggest that the cytoprotective action of Re^{II}-based CORMs may be attributed to both the extracellular release of CO and to antioxidative action of the molecules. None of the tested rhenium adducts interfered with mitochondrial respiration. Chemical and biochemical evidence indicate that the non-toxicity of the Re^{II}-based CORMs may be ascribed to the oxidation of the metal and formation in solution of the ReO_4^- anion which is among the least toxic of all of the rare inorganic compounds.[41] Current efforts are directed towards the elucidation of the molecular mechanisms underlying the cytoprotective effects of these species and to the synthesis of $Re^{II}(CO)_2$ conjugates of other naturally occurring molecules.

Experimental section

Chemicals and solvents were purchased from standard sources. All synthesis were performed under an inert N_2 atmosphere unless otherwise noted. ReCORM-**1** was synthesized according to literature.[40] Elemental analyses (EA) were performed on a Leco CHNS-932 elemental analyzer. IR spectra were recorded in a PerkinElmer Spectrum BX FT-IR spectrometer. Crystallographic data were collected at 183(2) K with Mo-Kα radiation (λ = 0.7107 Å) that was monochromated with help of a graphite monochromator on an Oxford Diffraction Xcalibur system with a Ruby detector. Suitable crystals were covered with oil (Infineum V8512), mounted on top of a glass fiber and immediately transferred to the diffractometer. The program suite CrysAlis^{Pro} was used for data collection, semi-empirical absorption correction and data reduction.[51] Structures were solved with direct methods using SIR97[52] and were refined by full-matrix least-squares methods on F^2 with SHELXL-97.[53] The structures were checked for higher symmetry with help of the program Platon.[54]

Analytical HPLC method

Instrument: MERCK HITACHI LaChrom with a D-7000 interface coupled with a Diode Array detector L-7455 and a pump L-7100 system. Column: Macherey-Nagel, EC250/3 Nucleosil 100-5 C18. Flow rate 0.5 mL min^{-1}. Absorbance monitored at 250 nm. Solutions: A: 0.1% trifluoroacetic acid in water; B: methanol. Chromatographic method: 0–5 min: isocratic flow of 75% A–25% B; 5–30 min: linear gradient to 100% B; 30–35 min: isocratic flow of 100% B; 35–40 min linear gradient to 75% A–25% B.

Computational details

Geometry optimizations as well as frequency calculations for all "small" molecules, were performed at the Density Functional level of theory with the Gaussian03 program package[55] using the hybrid B3LYP functional[56] in conjunction with the LanL2DZ basis set.[57–59] Pure basis functions (5d, 7f) were used in all calculations. Geometries were fully optimized without symmetry restrictions. The nature of the stationary points was checked by computing vibrational frequencies in order to verify true minima. The lowest 30 singlet excitation energies were computed by means of time-dependent DFT (TD-DFT) methodology[60–62] to simulate the absorption electronic spectra. Solvent effects were taken into account using the polarizable continuum model (PCM)[63] with water as solvent for the TD-DFT calculations. The molecular structures of B_{12}-ReCORM-**2** and B_{12}-ReCORM-**4** were optimized by the two-layer ONIOM method[64,65] using the DFT method B3LYP/LanL2DZ for the high layer and the molecular mechanics method UFF for the low layer (see description of the layers in the ESI†).[66]

Detection of CO release using the myoglobin assay

The release of CO from compounds B_{12}-ReCORM-**2** and B_{12}-ReCORM-**4** was assessed spectrophotometrically by measuring

the conversion of deoxymyoglobin (Mb) to carbonmonoxy myoglobin (MbCO) as previously reported.[30,31] A small aliquot of a freshly prepared concentrated aqueous solution of the selected Re^{II} complex was added to 1 ml of the Mb solution in phosphate buffer (0.1 M, pH 7.4, final concentrations: 30 μM for Re^{II} complex and Mb). Changes in the Mb spectra were recorded over time at 25 °C. The amount of MbCO formed was determined by measuring the absorbance at 540 nm (extinction coefficient = 15.4 M^{-1} cm^{-1}). The MbCO concentration was plotted over time and directly related to the equivalents of CO released from the compounds. The half-life of CO release from B_{12}-ReCORM-**2** and B_{12}-ReCORM-**4** was then estimated from the graphs. Control experiments were run under identical conditions but without addition of the metal complexes. All manipulations were performed under a pure N_2 atmosphere in a wet box.

Synthesis of B_{12}-ReCORM-2

Cyanocobalamin (10 mg, 7.4 μmol) and ReCORM-**1** (10 mg, 12 μmol) were added as solids to a round bottom flask. Methanol (6 mL) was added, stirring began and then the flask was lowered into an oil bath preheated to 50 °C. The temperature was then lowered to 40 °C within 1 h. After 1.5 h HPLC analysis showed a single B_{12} derivative. Heating was stopped and the solvent removed under reduced pressure. The resulting red powder was washed several times with CH_2Cl_2 (*ca.* 1.7 eq. of [Et4N]Br was thus recovered) and then acetone until washings were clear. Compound B_{12}-ReCORM-**2** was thus obtained as a red microcrystalline powder. Yield: 12.8 mg, 98%. HPLC analysis showed a single peak with a retention time of 21.9 min. Analytical data for B_{12}-ReCORM-**2**: ESI-MS analysis (positive mode) gave peaks at m/z = 1758.4 $[M+H^+]^+$ and 879.8 $[M+H^+]^{2+}$. I.R. (solid state, KBr, cm^{-1}): $\nu_{C\equiv N}$ 2184, $\nu_{C\equiv O}$ 1989, 1839.

Synthesis of 3

Cyanocobalamin (400 mg, 0.3 mmol) was dissolved in 10 mL of anhydrous DMSO and carbonyldiimidazole (CDI, 1 g, 6.2 mmol) was added. The solution was stirred and heated to 60 °C for 12 h. The solution was allowed to cool to room temperature (RT) and then slowly added to a rapidly stirring mixture of 300 mL 1 : 1 diethyl ether : chloroform. The red precipitate was collected by vacuum filtration, washed with 50 mL of acetone and vacuum dried. Yield: 395 mg. This product was not purified but used directly for the next reaction. 100 mg of the activated vitamin was dissolved in 5 mL of anhydrous DMSO and 4-picolylamine (100 μl, 1 mmol) was added. The solution was stirred at RT for 30 min and then slowly added to a rapidly stirring mixture of 150 mL 1 : 1 diethyl ether : chloroform. The red precipitate was collected by vacuum filtration, washed with 30 mL of acetone and vacuum dried. Yield: 109 mg. This precipitate consists of a mixture of products. Compound **3** was then purified by HPLC (retention time 15.5 min). Analytical data for **3**: yield 45 mg. ESI-MS analysis (positive mode) gave a single peak at m/z = 745.6 corresponding to $[M+H^+]^{2+}$. ^1H NMR, 500 MHz (D_2O, δ (ppm), aromatic region): 8.28 (s, 2H, py), 7.36 (s, 2H, py), 7.27 (s, 1H, B7), 7.12 (s, 1H, B2), 6.55 (s, 1H, B4), 6.33 (s, 1H, R1), 6.11 (s, 1H, C10). See also ESI.† I.R. (solid state, KBr, cm^{-1}): $\nu_{C\equiv N}$ 2134. Single crystals suitable for X-ray diffraction were grown by slow diffusion of acetone into an aqueous solution of the compound.

Synthesis of B_{12}-ReCORM-4

As described for B_{12}-ReCORM-**2** but starting from **3** instead of cyanocobalamin. Compound B_{12}-ReCORM-**4** was obtained as a red microcrystalline powder. Yield: 95%. HPLC analysis showed a single peak with a retention time of 25.3 min. Analytical data for B_{12}-ReCORM-**4**: ESI-MS analysis (positive mode) gave peaks at m/z = 1893.4 $[M+H^+]^+$ and 946.8 $[M+H^+]^{2+}$. I.R. (solid state, KBr, cm^{-1}): $\nu_{C\equiv N}$ 2184, $\nu_{C\equiv O}$ 1989, 1839.

In vitro model of "ischemia-reperfusion" stress of cardiomyocyte

Animal keeping, breeding and experimentation were reviewed, approved, and carried out in accordance with the Swiss animal protection laws and institutional guidelines. Neonatal rat cardiomyocytes (NRCs) were isolated from ventricular tissue of Wistar rat pups at postnatal day 3 by collagenase digestion followed by separation on Percoll density gradient as described elsewhere. The cells were maintained in culture for 4 days and then used for experiments. Conditions of ischemia-reperfusion were mimicked as follows. The "ischemic incubation medium" was glucose- and serum-free with its pH adjusted to 6.0. Immediately after replacing the normal cell culture medium by ischemic medium the NRCs were placed into the hypoxic incubators in which oxygen concentration was reduced to 1% for 16 h. Thereafter the medium was replaced by the one at pH 7.4 containing 5 mM glucose, 10% fetal calf serum (FCS). Reoxygenation and restoration of pH, glucose and FCS supplementation imitated "reperfusion" phase. Rhenium-based CORMs were added from the 30 mM stock solution prepared in DMSO to the cell culture medium immediately after the onset of "reperfusion" to a final concentration of 30 μM. Equal amounts of solvent were added to the control samples. The cells were placed into an incubator in the atmosphere of 95% air and 5% CO_2 for a further 9 h and then the percentage of dead cells was determined using a combined stating with Hoechst 33342 (0.1 μg ml^{-1}) and propidium iodide, PI (0.5 μg ml^{-1}). The resulting nuclear fluorescence was monitored using an inverted fluorescent microscope Axiovert 200 M equipped with DAPI (409 nm excitation/450 nm emission) and Cy3 (550 nm excitation/570 nm emission) filters. The total number of cells per field (Hoechst 33342-positive nuclei) and the number of dead cells (PI-positive nuclei) were counted for 5 fields per 4.5 cm^2 Petridish. Experiments were repeated 6–8 times with cells obtained in 3 independent isolations.

Effect of selected Re^{II}-based CORMs on oxygen consumption by NRCs and oxygen content on the cell-free culture medium

The effect of B_{12}-ReCORM-**2**, compounds ReCORM-**1** and ReCORM-**6** on the oxygen consumption by the NRCs was examined in cell culture medium at 37 °C using a high-resolution Oroboros Oxygraph-2k (Oroboros, Innsbruck, Austria). The Oxygraph is a two-chamber titration-injection respirometer with an oxygen detection limit of up to 0.5 pmol sec^{-1} mL^{-1}. Standardized instrumental and chemical calibrations were performed to correct for back-diffusion of oxygen into the chamber from the various components, leak from the exterior, oxygen consumption by

the chemical medium, and sensor oxygen consumption. Oxygen flux was resolved by software allowing nonlinear changes in the negative time derivative of the oxygen concentration signal (Oxygraph 2k, Oroboros, Innsbruck, Austria). Following 4 days of culture the cells were trypsinised, resuspended in the cell culture medium, and transferred into their respective respiratory chamber (~1 Mio of cells per chamber), which were then tightly sealed. Oxygen consumption in both chambers was monitored over time with the oxygen flux expressed as pmol/(sec·Mio cells). After basal oxygen consumption was established, either CORMs or DMSO alone was introduced into the chamber and the change in oxygen flux was measured as a function of time and CORM concentrations. Similar measurements were repeated for the CO-MRs added into the cell-free incubation medium to assess the effect of CORM addition on the oxygen levels in the cell culture medium. These experiments were performed as the some of the reactive CORM intermediates were suggested to interact with oxygen in the aqueous phase (see Scheme 3).

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45 **5**, **Crystal data**. $0.53(C_{63}H_{86}CoN_{14}O_{14}P)\cdot0.47(C_{66}H_{88}CoBrN_{14}O_{18}PRe)\cdot C_3H_6O\cdot6.53(H_2O)$, M = 1700.37, orthorhombic, a = 15.8806(2), b = 21.8255(3), c = 26.2738(4) Å, U = 9106.6(2) Å^3, T = 183 K, space group $P2_12_12_1$ (no.19), Z = 4, 79318 reflections measured, 20093 unique (R_{int} = 0.0603) which were used in all calculations. The final $wR(F_2)$ was 0.1884 (all data).
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U = 8961.1(4) Å^3, T = 183 K, space group $P2_12_12_1$ (no.19), Z = 2, 71958 reflections measured, 16995 unique (R_{int} = 0.1164) which were used in all calculations. The final $wR(F_2)$ was 0.1588 (all data).
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