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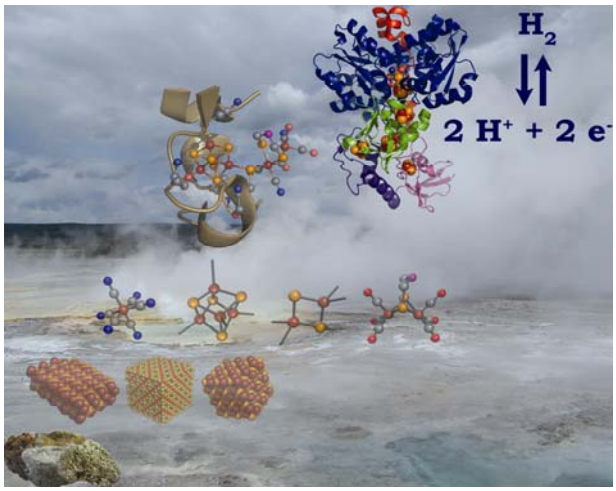

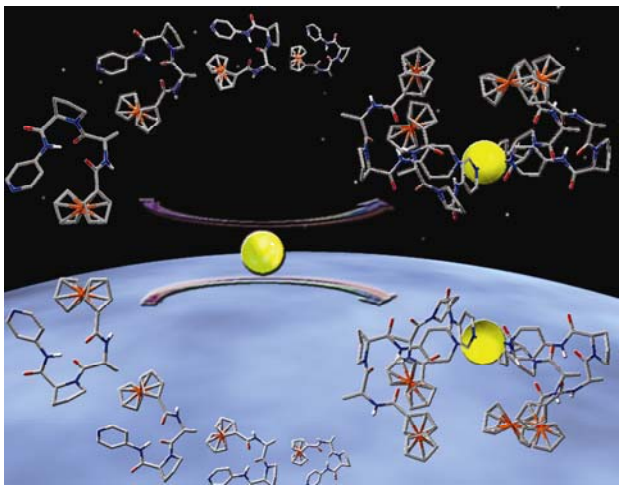

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Sonogashira and "Click" reactions for the *N*-terminal and side-chain functionalization of peptides with $[Mn(CO)_3(tpm)]^+$-based CO releasing molecules (tpm = tris(pyrazolyl)methane)†

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A recently identified photoactivatable CO releasing molecule (CORM) based on $[Mn(CO)_3(tpm)]^+$ was conjugated to functionalized amino acids and model peptides using the Pd-catalyzed Sonogashira cross-coupling and the alkyne–azide 1,3-dipolar cycloaddition ("Click reaction"). Both were found to be fully compatible with all functional groups present. The CORM–peptide conjugates were isolated in reasonable yield and high purity, as indicated by IR spectroscopy, ESI mass spectrometry and RP-HPLC. The myoglobin assay was used to demonstrate that they have CO release properties identical those of the parent compound. This work thus opens the way for a targeted delivery of CORMs to cellular systems.

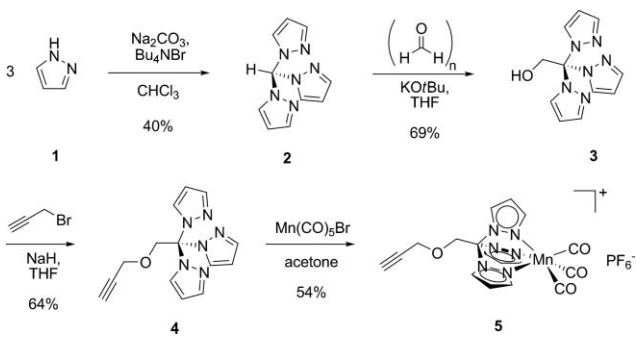
Introduction

In recent years, the physiological function of carbon monoxide (CO) in addition to that of nitric oxide (NO) as a small-molecule messenger has become the focus of intense research efforts.[1–3] To elucidate the biological mode of action of CO at the molecular level and for potential therapeutic applications,[4–6] there is a steadily growing interest in the use of CO releasing molecules (CORMs) as a stable solid storage form for carbon monoxide,[7] which is much more easy to handle and apply than the highly toxic gas itself. Transition-metal carbonyl complexes are a natural choice for the development of CORMs and a number of manganese, iron, molybdenum, and especially ruthenium carbonyl complexes have been studied in this context.[8] Most of them, however, are relatively simple compounds which allow for little structural variation, such as, for example, the commonly employed $[RuCl(glycinato)(CO)_3]$ (CORM-3).[9] Only very recently, some pyrone complexes of iron and molybdenum carbonyls have been reported by Motterlini *et al.*, in which substituents on the η^1- or η^4-coordinated pyrone ring can be modified to influence the CO release properties.[10–13] In addition, we have identified a novel $[Mn(CO)_3(tpm)]^+$-based CORM which exhibits photoinduced release of two carbonyl ligands and is efficiently taken up by HT-29 human colon carcinoma cells. While the compound is inactive in the dark at up to 100 μM, its irradiation leads to a very efficient reduction of cell biomass, comparable to that induced by established anticancer agent 5-fluorouracil (5-FU) in clinical use for many years.[14] For a targeted delivery of our novel CORM, it was envisioned that the functionalization of the tris(pyrazolyl)methane (tpm) ligand with peptides might enable a specific uptake in cancer cells.[15–20] Since the tpm moiety is not stable under the normal conditions of Fmoc-based solid-phase peptide synthesis (SPPS) we decided to explore a "post"-labelling strategy in which the tpm–manganese tricarbonyl moiety is attached to the peptide after synthesis and purification by conjugation methods orthogonal to the functional groups present in the peptide and the CORM. In the present work, the utility of the Pd-catalyzed Sonogashira cross-coupling[21] and the alkyne–azide 1,3-dipolar cycloaddition ("Click reaction")[22] for such modifications was thus explored on functionalized amino acids and model peptides.

Results and discussion

Synthesis and characterization of model compounds

As the alkynyl component for either the Sonogashira cross-coupling reaction or a 1,3-dipolar cycloaddition, tris-2,2,2-(pyrazol-1-yl)ethoxypropargyl ether (**4**) and its corresponding manganese(I) tricarbonyl complex **5** were synthesized according to a procedure reported by Reger *et al.* (Scheme 1).[23] The IR spectrum of the complex shows the characteristic two CO vibrations at 2050 and 1951 cm^{-1}, as expected in C_{3v} symmetry. The ^1H and ^{13}C NMR



Scheme 1 Synthesis of tpm-alkynyl complex $[Mn(CO)_3(tpm\text{-}L1)]$ **5** for Sonogashira cross-coupling and alkyne–azide "Click" reactions.

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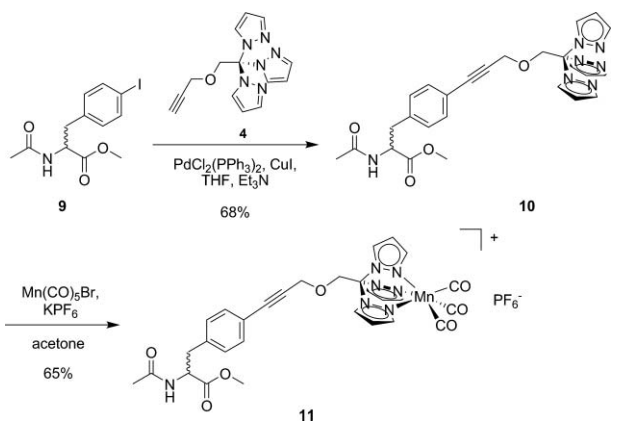
† Electronic supplementary information (ESI) available: Synthesis of ligand precursors, additional HPLC traces, IR and mass spectra of the peptide conjugates. See DOI: 10.1039/b819091g

spectra of the ligand as well as the complex are in full accordance with the expected structure.

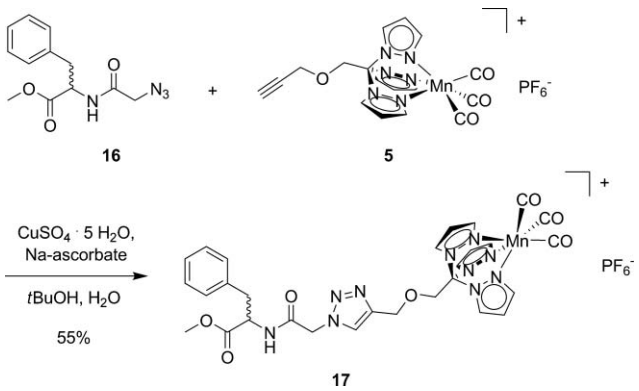
To test reaction conditions for the synthesis of $[Mn(CO)_3(tpm)]^+$-peptide conjugates, model compounds based on single amino acids were synthesized first. For this purpose, *N*-acetyl-4-iodo-DL-phenylalaninemethylester (**9**) was prepared as the iodoarene component for a Sonogashira cross-coupling reaction in three steps from DL-phenylalanine (**6**), which was regioselectively iodinated in the *para*-position of the phenyl ring (Scheme S1, ESI†).

Subsequently, the carboxy group was esterified and the amino group acetylated in order to improve the solubility in solvents suitable for Sonogashira cross-coupling reactions.

The final Sonogashira cross-coupling of compounds **4** and **9** was performed under "classical" conditions with $PdCl_2(PPh_3)_2$ as the pre-catalyst for an *in situ* generated Pd(0) species and copper(I) iodide as the co-catalyst (Scheme 2 and S2, ESI†). The reaction proceeded to completion within 24 h, as indicated by the disappearance of the ^{1}H NMR signal of the terminal alkynyl proton at 2.50 ppm. The product tpm-L1 **10** was obtained in good yield (68%) and fully characterized by H,H-COSY, HMBC and HSQC NMR spectroscopy. The reaction of ligand **10** with manganese pentacarbonyl bromide in acetone and subsequent precipitation with potassium hexafluorophosphate yielded the model complex $[Mn(CO)_3(tpm\text{-}L2)]PF_6$ (**11**). The ESI$^+$-mass spectrum exclusively shows the $[M - PF_6]^+$ peak of the desired product at $m/z = 640$ (Fig. S1, ESI†). The IR spectrum of complex **11** shows three bands for the CO vibrations of the metal tricarbonyl moiety at 2049, 2019 and 1934 cm^{-1} (inset in Fig. S1, ESI†). Since the IR spectrum was recorded with an ATR accessory on a pure solid sample, this is due to solid-state effects, which lead to a lowering of the symmetry at the manganese center. The solution ^{13}C NMR spectrum, on the other hand, shows only one signal for the three equivalent CO ligands at 219.3 ppm. To explore an alternative conjugation method *via* a 1,3-dipolar cycloaddition of an azide and the terminal alkyne group of **5** ("Click reaction"), DL-phenylalaninemethyl ester hydrochloride (**13**) was coupled to azidoacetic acid (**15**) with isobutylchloroformate as the coupling reagent to form azido compound **16** (Scheme S3, ESI†). The ^{1}H and ^{13}C NMR spectra of the product **16** show all expected signals. The IR spectrum displays the characteristic azido vibrational band at 2101 cm^{-1}. The cycloaddition reaction between **16** and $[Mn(CO)_3(tpm\text{-}L1)]PF_6$ (**5**) was performed in a *tert*-butanol–water mixture (1 : 1) using copper(II) sulfate pentahydrate as the pre-catalyst and sodium ascorbate as the *in situ* reductant to generate the catalytically active copper(I) species (Scheme 3).



Scheme 2 Synthesis of $[Mn(CO)_3(tpm\text{-}L2)]^+$ model conjugate **11** *via* Sonogashira cross-coupling.



Scheme 3 Synthesis of the peptide conjugate model compound $[Mn(CO)_3(tpm\text{-}L3)]PF_6$ (**17**) *via* 1,3-dipolar cycloaddition.

The progress of the reaction could easily be monitored by the low-field shift of the alkynyl proton ^{1}H NMR signal from 3.83 ppm in (**5**) to 8.29 ppm in (**17**) and the disappearance of the azido vibrational band in the IR spectrum. The model compound was obtained in moderate yield (55%). In the ESI$^+$ mass spectrum only the peak for the molecular ion at $m/z = 683$ is found (Fig. 1). Its IR spectrum shows the two CO vibrational bands of the manganese tricarbonyl moiety at 2049 and 1938 cm^{-1} (inset in Fig. 1).

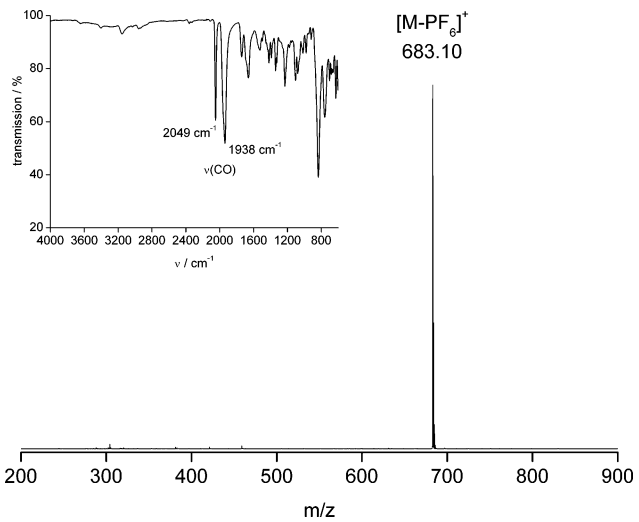


Fig. 1 ESI$^+$ mass spectrum and (inset) ATR-IR spectrum of $[Mn(CO)_3(tpm\text{-}L3)]PF_6$ (**17**).

Synthesis and characterization of peptide conjugates

Since the conditions chosen for the synthesis of functionalized amino acids *via* Sonogashira cross-coupling as well as 1,3-dipolar cycloaddition reactions were found to be compatible with all functional groups present, they were then applied to the synthesis of peptide conjugates. The five amino acid sequence Thr-Phe-Ser-Asp-Leu was chosen as the peptide component. This sequence is part of the transactivation domain of the tumor suppressor protein

p53 with the regulatory protein MDM2 (murine double minute 2). The latter is an E3-ubiquitin ligase which regulates the activity of the p53 transcription factor. Overexpression of MDM2 reduces the p53 activity and thus cell cycle control is lost, allowing further division of cells with damaged DNA, facilitating the development of cancer.[24] Here, however, it was mainly selected to provide a number of side-chain functional groups which might interfere with the coupling partners and reactants used. In order to introduce suitable linkers for the two conjugation methods, the pentapeptide was functionalized with iodoarene as well as azido groups at the *N*-terminus and in the side chain (Fig. 2).

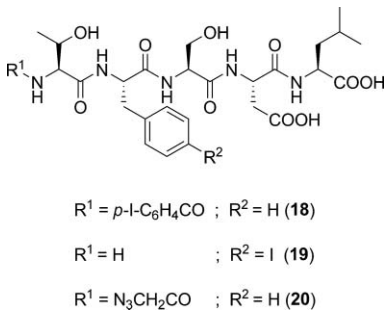
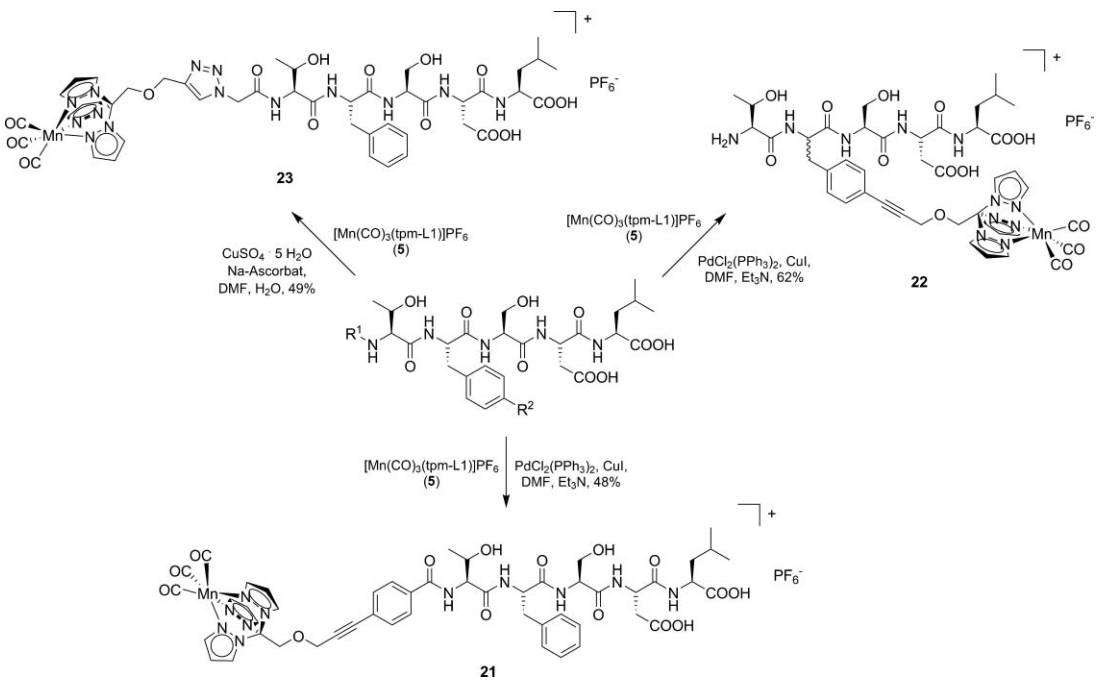


Fig. 2 Functionalized pentapeptides **18** to **20** synthesized.

The solid-phase synthesis (SPPS) of the functionalized peptides **18** to **20** was performed under microwave irradiation to speed up reaction times and reduce the amount of by-products formed.[25] Due to a potential decomposition of the azido moiety under SPPS conditions, the final coupling of azidoacetic acid (**15**) in the synthesis of peptide **20** was carried out manually outside the microwave reactor. All peptides were characterized by ESI mass spectrometry revealing a high purity of the products since only the corresponding $[M + H]^+$ peaks were detected. Therefore, no further purification steps were carried out before use in the next step. For the synthesis of peptide conjugates *via* Sonogashira cross-coupling reactions of *N*-terminal and side-chain iodoarene functional groups, **18** and **19** were successfully coupled with $[Mn(tpm\text{-}L1)(CO)_3]PF_6$ (**5**) containing an alkynyl-substituted tpm ligand in a mixture of dimethylformamide and triethylamine under the same conditions as successfully employed for the model compounds (Scheme 4). The *N*-terminally functionalized conjugate **21** was obtained in moderate yield (48%) but RP-HPLC analysis reveals a high purity (94%). This was confirmed by the ESI mass spectrum, which only shows the $[M - PF_6]^+$ peak of the product at $m/z = 1104$ (Fig. S2, ESI†). The IR spectrum displays the expected vibrational bands of the carbonyl ligands at 2048 and 1944 cm^{-1} (Fig. S3, ESI†) and indicates their integrity under the coupling conditions. The analogous coupling reaction with the side chain-functionalized peptide **19** gave conjugate **22** in somewhat better yield (62%) but slightly lower purity (91%, Scheme 4). The ESI^+ mass spectrum again shows the expected peak of the product at $m/z = 1000$ (Fig. S4, ESI†) and the vibrational bands of the carbonyl ligands are found in the IR spectrum at 2049 and 1942 cm^{-1} (Fig. S5, ESI†). The 1,3-dipolar cycloaddition of **20** and $[Mn(CO)_3(tpm\text{-}L1)]PF_6$ (**5**) using the catalyst combination already tested was successful as well. In contrast to the conditions originally used in the synthesis of model compound **17**, dimethylformamide was used instead of *tert*-butanol due to a better solubility of the starting materials (Scheme 4). Peptide conjugate **23** was obtained in moderate yield (49%) but with an excellent purity of >98%. The significantly reduced retention time in the RP-HPLC chromatogram, compared to the two other peptide conjugates synthesized *via* Sonogashira cross-coupling might be due to the presence of the 1,2,3-triazole moiety. In the ESI mass spectrum, the product peak $[M - 2H - PF_6]^-$ at $m/z = 1083$ is only found in the negative mode (Fig. 3). The



Scheme 4 Synthesis of $[Mn(CO)_3(tpm)]^+$–peptide conjugates **21–23** *via* Sonogashira cross-coupling and 1,3-dipolar cycloaddition.

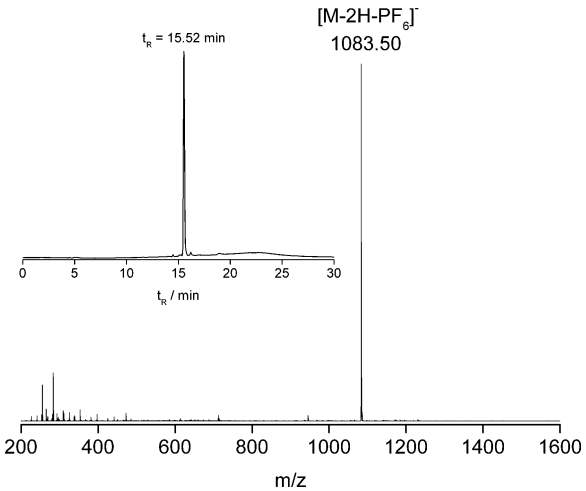


Fig. 3 ESI⁻ mass spectrum and (inset) analytical HPLC chromatogram (254 nm) of peptide conjugate **23**.

IR spectrum displays the CO vibration bands of the manganese tricarbonyl moiety at 2048 and 1941 cm^{-1} (Fig. 4) while no trace of the azido vibration is found.

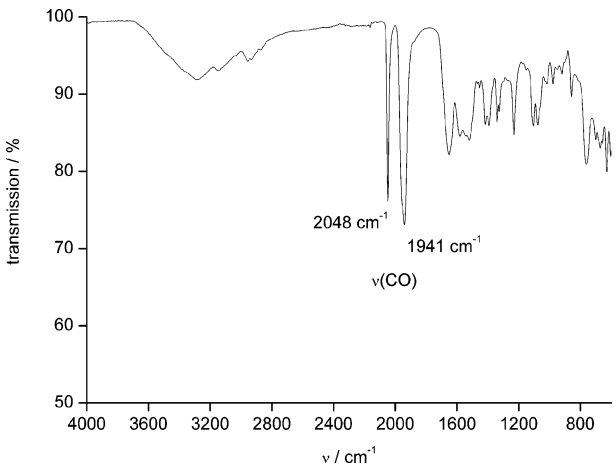


Fig. 4 ATR-IR spectrum of peptide conjugate **23**.

CO release studies on peptide conjugate 23

To test whether the CO release properties of the $[Mn(CO)_3(tpm)]^+$ moiety remain unaltered by the peptide conjugation, compound **23** was added to a buffered aqueous solution of horse skeletal myoglobin (MbFe(II)), freshly reduced with excess sodium dithionite under nitrogen. When kept in the dark, no spectral changes were observed over 60 min, indicating that the conjugate is stable under these conditions on that timescale. This was also confirmed by ESI-MS measurements. Upon irradiation of the mixture at 365 nm, however, characteristic changes in the band intensities were observed in the Q band region, which indicate a conversion of the MbFe(II) with $\lambda_{max} = 557$ nm to the MbFeCO complex, which has maxima at 542 and 577 nm (Fig. 5). Taking into account the molar extinction coefficient of MbFeCO and correcting for the amount of Mb Fe(II) still present, it was found that approximately 1.7 mol of CO were liberated per mol of **23**. This compares rather well with the data obtained for the parent $[Mn(CO)_3(tpm)]^+$ complex.[14] The slightly lower number of CO equivalents released might be due to a somewhat lower accuracy in the concentration determination of **23**, since the hydration state of the peptide is not known even after extended lyophilization.

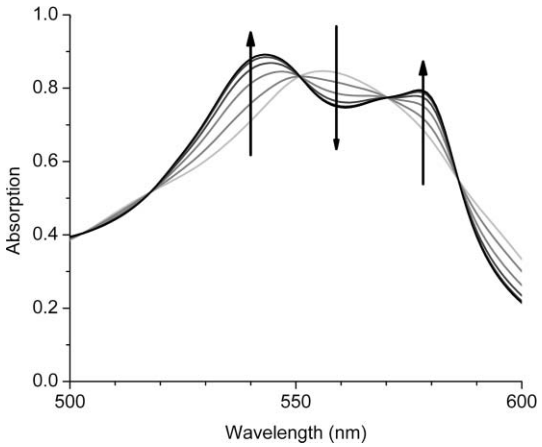


Fig. 5 UV/Vis spectral changes in the Q band region of a solution of reduced horse skeletal muscle myoglobin (70 μmol L^{-1}) and peptide conjugate **23** (20 μmol L^{-1}) in 0.1 M phosphate buffer upon irradiation at 365 nm ($t = 0$ to 130 min).

Conclusions

In the present work, we have shown that an alkyne-substituted derivative of the recently identified photoactivatable CORM $[Mn(CO)_3(tpm)]^+$ can be conjugated to azide- and iodoarene-functionalized model compounds *via* 1,3-dipolar cycloaddition or Sonogashira cross-coupling reactions, respectively. The conditions used were fully compatible with all functional groups present and thus applied to conjugate the CORM to short peptides carrying either a *N*-terminal iodoarene or azide moiety as well as a 4-iodophenylalanine at one position in the sequence. The CORM-peptide conjugates were isolated in reasonable yield and high purity, as indicated by IR spectroscopy, ESI mass spectrometry and RP-HPLC. With the myoglobin assay, it was demonstrated that the peptide conjugation does not alter the CO release properties of the $[Mn(CO)_3(tpm)]^+$ moiety when compared to the parent compound. Currently, we are exploring the utility of these bioconjugates for the targeted delivery of manganese tricarbonyl-based CORMs to cellular systems.

Experimental

General procedures

Reactions were carried out in oven-dried Schlenk glassware under an atmosphere of pure nitrogen when necessary. 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 (^1H at 200.13 and 400.13 MHz, respectively; ^{13}C at 50.33 and 100.62 MHz). Chemical shifts δ in ppm indicate a downfield shift relative to tetramethylsilane (TMS) and were referenced relative to the signal of the solvent.[26] 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 Bruker Esquire 6000 (ESI) and VG Autospec (FAB) instruments, only characteristic fragments are given for the most abundant isotope peak. The solvent flow rate for the ESI measurements was 4 µl/min 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. IR spectra were recorded on pure solid samples with 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. The analytical HPLC measurements were performed on a Varian ProStar instrument using a Dynamax RP analytical column (C_{18} microsorb 60 Å, diameter 10 mm, 250 mm length) with a mixture of water and acetonitrile containing 0.1% v/v trifluoroacetic acid as the eluent, using a linear gradient of 5–95% acetonitrile over 30 min at a flow rate of 1 mL min^{-1}. The myoglobin assay was carried out in a quartz cuvette ($d = 1$ cm) with a Jasco V-670 UV/Vis spectrometer as described below.

Microwave-assisted solid-phase peptide synthesis

All peptides were prepared on a CEM Liberty peptide synthesizer on a 0.25 mmol scale, using the Fmoc-strategy and a pre-loaded Fmoc-Leu–Wang resin as solid support under microwave-irradiation in all steps. Deprotection of the Fmoc-protected amino acids was done with a solution of 20% piperidine in dimethylformamide. For each coupling step, four equivalents of amino acid (0.2 M in DMF) and coupling reagent (HOBT/TBTU, 0.45 M in DMF) were used. Diisopropylethylamine (DIPEA) served as the activator base. After the last coupling step, the resin was taken out of the microwave reactor and cleavage of the peptide from the solid support was performed in a filter syringe at room temperature, using a solution of TFA–TIS–H_2O (90 : 5 : 5).

The peptide was then isolated by precipitation with cold diethyl ether (–20 °C) and repeated cycles of washing, centrifugation and decanting. The remaining residue was dissolved in a acetonitrile–water mixture and lyophilized, yielding the peptides as essentially white solids. Different from the above procedure, the final coupling of azidoacetic acid (**15**) was performed in a filter syringe at RT using a solution of azidoacetic acid–HOBT–TBTU (each 0.4 M) in DMF with DIPEA as the activator base (0.3 M) over 90 min. After excessive washing with DMF and dichloromethane, the peptide was cleaved from the resin as described above.

Synthetic procedures

$[Mn(CO)_3(tpm\text{-}L1)]PF_6$ (5). Tris-2,2,2-(pyrazol-1-yl)ethoxypropargyl ether (**4**) (1.00 g, 3.50 mmol) and manganese pentacarbonyl bromide (0.94 g, 3.40 mmol) were dissolved in anhydrous acetone (30 mL) and heated to reflux for 6 h under a nitrogen atmosphere with exclusion of light. The yellow precipitate was filtered off, re-dissolved in methanol and an aqueous solution of potassium hexafluorophosphate (571 mg, 3.40 mmol) was added. The yellow precipitate was filtered off and dried under vacuum. Yield: 54% (1.03 g, 1.82 mmol). Elemental analysis (%): calc. for $C_{17}H_{14}F_6MnN_6O_4P$: C 36.06, H 3.49, N 14.84, found: C 35.71, H 3.42, N 14.69; MS (ESI^+, CH_3OH): m/z 421 $[M - PF_6]^+$; IR (ATR, cm^{-1}): 3306 (w) ν(CC–H), 2130 (w) ν(C≡C), 2050 (s) ν(*fac*-C=O), 1951 (s) ν(*fac*-C=O); ^{1}H NMR (400.13 MHz, DMSO-d_6): δ 8.70 (s, br, 3H, H(3-pz)), 8.59 (s, br, 3H, H(5-pz)), 6,79 (s, br, 3H, H(4-pz)), 5.60 (s, 2H, $OCH_2C(pz)_3$), 4.71 (d, 2H, OCH_2CC, $^4J = 2.0$ Hz), 3.82 (t, 1H, C≡CH, $^4J = 2.0$ Hz) ppm; ^{13}C NMR (100.62 MHz, DMSO-d_6): δ 219 (C=O), 148.00 ($C_{3\text{-}pz}$), 139.07 ($C_{5\text{-}pz}$), 108.66 ($C_{4\text{-}pz}$), 82.56 ($C_{q\text{-}tpm}$), 79.87 (*C*≡C), 78.18 (C≡*C*), 64.99 (OCH_2–$C_{q\text{-}tpm}$), 58.63 (OCH_2–C≡C) ppm.

$[Mn(CO)_3(tpm\text{-}L2)]PF_6$ (11). Manganese pentacarbonyl bromide (126 mg, 0.47 mmol) und tpm-L2 (**10**) (234 mg, 0.47 mmol) were dissolved in anhydrous acetone (15 mL) and heated to reflux for 4.5 h under a nitrogen atmosphere with exclusion of light. After cooling to RT, the solvent was removed *in vacuo* and the yellow residue redissolved in methanol. Upon addition of an aqueous solution of sodium hexafluorophosphate (87.0 mg, 0.47 mmol), a bright yellow precipitate formed which was dissolved again by heating and finally crystallized at +4 °C. Yield: 65% (240 mg, 0.31 mmol). Elemental analysis (%): calc. for $C_{29}H_{34}F_6MnN_7O_7P{\cdot}3.5H_2O$: C 41.05, H 4.04, N 11.55, found: C 40.85, H 3.01, N 11.14; MS (ESI^+, acetone): m/z 640 $[M - PF_6]^+$; IR (ATR, cm^{-1}): 2049 (s) ν(*fac*-C=O), 2019 (s) ν(*fac*-C=O), 1934 (s) ν(*fac*-C=O), 1738 (s) ν(C=O, ester), 1661 (s) ν(C=O, amide); ^{1}H NMR (400.13 MHz, DMSO-d_6): δ 8.90 (d, 1H, NH, $^3J_{CH,NH} = 7.7$ Hz); 8.68 (d, br, 3H, H(3-pz), $^3J = 2.5$ Hz), 8.62 (d, br, 3H, H(5-pz), $^3J = 2.5$ Hz), 8.14 (s, br, 2H, H-$Ar_{2,6}$), 7.96 (s, br, 2H, H-$Ar_{3,5}$), 6.77 (pseudo t, 3H, H(4-pz), $^3J = 2.5$ Hz), 5.66 (s, 2H, $OCH_2C(pz)_3$), 4.53 (ddd, 1H, H-α, $^3J_{CH,NH} = 7.7$ Hz, $^3J_{\alpha,\beta} = 8.8$ Hz, $^3J_{\alpha,\beta'} = 5.8$ Hz), 5.13 (s, 2H, C≡C–CH_2), 3.60 (s, 3H, OCH_3), 3.07 (dd, 1H, H-β, $^3J_{\alpha,\beta} = 8.8$ Hz, $^2J_{\beta,\beta'} = 13.8$ Hz), 2.95 (dd, 1H, H-β′, $^3J_{\alpha,\beta'} = 5.8$ Hz, $^2J_{\beta,\beta'} = 13.8$ Hz), 2.74 (s, 3H, NHCO–CH_3) ppm; ^{13}C NMR (100.62 MHz, DMSO-d_6): δ 219.29 (C=O), 171.44 (C=O, ester), 165.41 (C=O, amide), 141.80 ($C_{3\text{-}pz}$), 136.65 ($C_{1\text{-}Ar}$), 129.01 ($C_{2,6\text{-}Ar}$), 128.26 ($C_{5\text{-}pz}$), 126.61 ($C_{3,5\text{-}Ar}$), 126.19 ($C_{4\text{-}Ar}$), 108.62 ($C_{4\text{-}pz}$), 82.62 ($C_{q\text{-}tpm}$), 65.53 (Ar–*C*≡C), 64.07 (Ar–C≡*C*), 53.74 (OCH_2-$(pz)_3$), 51.92 (C≡C–CH_2–O), 51.28 (C_α), 36.70 (OCH_3), 35.71 (C_β), 30.71 (NHCO–CH_3) ppm.

$[Mn(CO)_3(tpm\text{-}L3)]PF_6$ (17). *N*-Azidoacetyl-DL-phenylalaninemethyl ester (**16**) (100 mg, 0.38 mmol) and [Mn(tpm-L1)$(CO)_3$]PF_6 (**5**) (213 mg, 0.38 mmol) were suspended in a 1 : 1 mixture of *tert*-butanol and water (12 mL). Then, sodium ascorbate (7.5 mg, 0.038 mmol, 10 mol%) and copper(II) sulfate pentahydrate (1 mg, 0.0038 mmol, 1 mol%) were added and the suspension stirred at room temperature under a nitrogen atmosphere for 48 h. The clear solution was poured into ice-water (50 mL) and the light yellow precipitate formed was separated by centrifugation, dissolved in acetonitrile and lyophilized, yielding a yellow hygroscopic solid. Yield: 55% (172 mg, 0.21 mmol). Elemental analysis (%): calc. for $C_{29}H_{28}F_6MnN_{10}O_7P$: C 42.04, H 3.41, N 16.91, found: C 42.41, H 3.77, N 16.74; MS (ESI^+, CH_3OH): m/z 683 $[M - PF_6]^+$; IR (ATR, cm^{-1}): 3403 (w), 3153 (w), 2957 (w), 2049 (s) ν(*fac*-C=O), 1938 (s) ν(*fac*-C=O), 1740 (m) ν(C=O, ester), 1661 (m) ν(C=O, amide); ^{1}H NMR (400.13 MHz, DMSO-d_6): δ 8.69 (s, br, 3H, H(3-pz)), 8.64 (s, br, 3H, H(5-pz)), 8.32 (d, 1H, H-Ar_4, $^3J = 5.7$ Hz), 8.29 (s, 1H, triazolyl-H), 7.47 (d, br, 2H, H-$Ar_{(2,6)(3,5)}$, $^3J = 5.7$ Hz), 7.26 (d, br, 2H, H-$Ar_{(2,6)(3,5)}$, $^3J = 5.7$ Hz), 6.78 (s, br, 3H, H(4-pz)), 5.69 (s, 2H, CH_2), 4.94 (s, 2H, CH_2), 4.46 (s, br, 1H, H-α), 3.58 (s, 2H, CH_2), 3.02 (m, br, 1H, H-β), 2.88 (m, br, 1H, H-β′), 1.76 (s, 3H, OCH_3) ppm; ^{13}C NMR (100.62 MHz, DMSO-d_6): δ 219.03 (C=O), 147.68 ($C_{3\text{-}pz}$), 138.43 ($C_{5\text{-}pz}$), 131.17, 129.09, 119.19, 108.80 ($C_{4\text{-}pz}$), 108.34, 87.70,

83.25, 82.42, 78.85, 64.63 (OCH_2-$C_{q\text{-}tpm}$), 58.96 (OCH_2-triazolyl), 52.91 (C_α), 51.52, 36.26 (C_β), 21.88 ppm.

Ibz-Thr-Phe-Ser-Asp-Leu-OH (Ibz-p53td-OH) (18). The peptide was prepared on a 0.25 mmol scale on a Fmoc-Leu–Wang resin (385 mg, 0.65 mmol g^{-1}) using the amino acids Fmoc-Thr(*t*Bu)-OH, Fmoc-Phe-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Asp(O*t*Bu)-OH and *p*-iodobenzoic acid under the conditions described above. Cleavage time: 3.5 h. Yield: 74% (150 mg, 0.179 mmol). RP-HPLC: t_R = 16.28 min; MS (ESI$^+$, CH_3CN–H_2O): *m*/*z* 812 $[M + H]^+$, 834 $[M + Na]^+$, 850 $[M + K]^+$; IR (ATR, cm^{-1}): 3273 (m), 3080 (m), 2961 (m), 1786 (m), 1716 (s), 1629 (s), 1536 (s), 1215 (s), 1155 (s).

H-Thr-Phe(I)-Ser-Asp-Leu-OH (H-p53td[Phe^2(I)]-OH) (19). The peptide was prepared on a 0.25 mmol scale on a Fmoc-Leu–Wang resin (385 mg, 0.65 mmol g^{-1}) using the amino acids Fmoc-Thr(*t*Bu)-OH, Fmoc-Phe(I)-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Asp(O*t*Bu)-OH and azidoacetic acid under the conditions described above. Cleavage time: 2 h. Yield: 60% (106 mg, 0.15 mmol). MS (ESI$^+$, CH_3OH): *m*/*z* 708 $[M + H]^+$; IR (ATR, cm^{-1}): 3282 (m), 3078 (m), 2959 (m), 1639 (s), 1523 (s), 1187 (s), 1139 (s).

N_3Ac-Thr-Phe-Ser-Asp-Leu-OH (N_3Ac-p53td-OH) (20). The peptide was prepared on a 0.25 mmol scale on a Fmoc-Leu–Wang resin (385 mg, 0.65 mmol g^{-1}) using the amino acids Fmoc-Thr(*t*Bu)-OH, Fmoc-Phe-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Asp(O*t*Bu)-OH under the conditions described above. Cleavage time: 3 h. Yield: 56% (90.5 mg, 0.14 mmol). MS (ESI$^-$, CH_3OH): *m*/*z* 663 $[M - H]^-$; IR (ATR, cm^{-1}): 3282 (m), 2961 (m), 2111 (s) $\nu(N_3)$, 2070 (m), 1634 (s) ν(C=O, amide).

$[Mn(CO)_3(tpm\text{-}CH_2OCH_2\text{–}C{\equiv}C\text{–}C_6H_4CO\text{-}p53td\text{-}OH)]PF_6$ (21). Ibz-p53td-OH (**18**) (30 mg, 37 μmol) and $[Mn(CO)_3$(tpm-L1)$]PF_6$ (**5**) (21 mg, 37 μmol) were dissolved in dimethylformamide (1 mL) and triethylamine (0.5 mL) and the solution was degassed by two "freeze–pump–thaw" cycles. Subsequently, copper(I) iodide (1 mg, 5.3 μmol, 12 mol%) and *cis*-dichlorobis(triphenylphosphine)palladium(II) (10.1 mg, 14 μmol, 2 mol%) were added and the clear yellow solution stirred for 20 h at RT under a nitrogen atmosphere protected from light. For purification, the brownish solution was absorbed on a short reversed phase column (Waters C_{18}-SepPak, 5 g) and the conjugate eluted with water. After lyophilization of the eluate, the product was obtained as pale yellow solid. Yield: 48% (22.0 mg, 0.018 mmol). RP-HPLC: t_R = 17.20 min; MS (ESI$^+$, CH_3OH): *m*/*z* 1104 $[M - PF_6]^+$; IR (ATR, cm^{-1}): 3290 (m) ν(OH), 2048 (s) ν(*fac*-C=O), 1944 (s) ν(*fac*-C=O).

$[Mn(CO)_3$(H-p53td[Phe^2(–C≡C–CH_2OCH_2-tpm)]-OH)$]PF_6$ (22). H-p53td[Phe^2(I)]-OH (**19**) (30 mg, 42 μmol) and $[Mn(CO)_3$(tpm-L1)$]PF_6$ (**5**) (24 mg, 42 μmol) were dissolved in dimethylformamide (1 mL) and triethylamine (0.5 mL) and the solution was degassed by two "freeze–pump–thaw" cycles. Then, copper(I) iodide (1 mg, 5.3 μmol, 12 mol%) and *cis*-dichlorobis(triphenylphosphine)palladium(II) (1 mg, 1.4 μmol, 3 mol%) were added under a nitrogen atmosphere and the mixture was degassed again. The clear yellow solution was stirred for 14 h at ambient temperature protected from light. For purification, the brownish solution was absorbed on a short reversed phase column (Waters C_{18}-SepPak, 5 g) and the conjugate eluted with water. After lyophilization, the product was obtained as a pale yellow solid. Yield: 62% (30.0 mg, 0.026 mmol). RP-HPLC: t_R = 17.01 min; MS (ESI$^+$, CH_3OH): *m*/*z* 1000 $[M - PF_6]^+$; IR (ATR, cm^{-1}): 3278 (m) ν(OH), 2049 (s) ν(*fac*-C=O), 1942 (s) ν(*fac*-C=O).

$[Mn(CO)_3$(tpm-CH_2OCH_2–C≡C–CH_2–$(C_2HN_3)CH_2$CO-p53td-OH)$]PF_6$ (23). N_3Ac-p53td-OH (**20**) (30 mg, 45 μmol) and $[Mn(CO)_3$(tpm-L1)$]PF_6$ (**5**) (26 mg, 45 μmol) were dissolved in a mixture of dimethylformamide (0.75 mL) and water (0.75 mL) and sodium ascorbate (2 mg, 10 μmol, 20 mol%) and copper(II) sulfate pentahydrate (0.5 mg, 2 μmol, 4 mol%) were subsequently added and the mixture was left stirring for 24 h while protected from light. The clear yellow solution was loaded on a reversed phase column (Waters C_{18}-SepPak, 5 g), washed with water (3 × 10 mL) and the peptide conjugate then was eluted with acetonitrile as a yellow band. Lyphilization yielded the product as a pale yellow solid. Yield: 49% (27 mg, 0.02 mmol). RP-HPLC: t_R = 15.52 min; MS (ESI$^-$, CH_3OH): *m*/*z* 1083 $[M - 2H - PF_6]^-$; IR (ATR, cm^{-1}): 3291 (m) ν(OH), 3150 (m), 2957 (m), 2048 (s) ν(*fac*-C=O), 1941 (s) ν(*fac*-C=O).

Myoglobin assay

In a quartz cuvette, horse skeletal muscle myoglobin 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 735 μl. To this solution, 15 μl of peptide conjugate **23** dissolved in 0.1 M phosphate buffer was added to give a final concentration of 20 μM of peptide conjugate and 70 μM of myoglobin with $A_{557\,nm}$ <1. Solutions were first kept in the dark for 60 min and then irradiated for up to 130 min under nitrogen with a UV hand lamp at 365 nm, positioned perpendicular to the cuvette at a distance of 12 cm. Irradiations were interrupted in regular intervals to take UV/Vis spectra on a Jasco V-670 spectrophotometer.

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