B712886J *Chem. Commun.*: Supplementary Information

Synthesis and Cytotoxicity of a Cobaltcarbonyl-Alkyne Enkephalin Bioconjugate

Merja A. Neukamm, Antonio Pinto and Nils Metzler-Nolte*

Department of Chemistry and Biochemistry, University of Bochum
Universitätsstrasse 150; 44801 Bochum, Germany
Fax: ++49 (0)234 - 32 14378; E-Mail: nils.metzler-nolte@ruhr-uni-bochum.de

CONTENT

I. Experimental Section: Materials and Methods

a) General remarks. ESI mass spectra were measured on a Bruker Esquire 6000. NMR data were measured on Bruker DRX 600 and DRX 400 instruments. RP-HPLC was performed using a Varian Pro Star PDA detector (model 330), a Varian solvent delivery system (model 210) and an analytical C-18 Microsorb (4.6 mm x 250 mm, 60 Å / 8 µm; Dynamax, Varian) or semipreparative C-18 Microsorb (10 mm x 250 mm, 60 Å / 8 µm; Dynamax, Varian) column. Water (A) and acetonitrile (B) were used as solvents, both containing 0.1 % TFA. The flow rate was 1 ml min^{-1} (analytical) or 4 ml min^{-1} (semipreparative) and peaks were detected at 254 mm and 220 nm. Typically, gradients were linear from 5 % -> 95 % (B) over 18 min.

b) Analytical data for Compounds 1, 2, and 3

1: R_t = 12.2 min, MS (ESI^-, MeOH): *m/z* 605 $[M-H]^-$, ^1H NMR (MeOH-d_4, 600.13 MHz): 7.31 (m, $\mathbf{H}_{o,m,\text{Ar, Phe}}$), 7.25 (m, $\mathbf{H}_{p,\text{Ar, Phe}}$) 7.08 (d, $\mathbf{H}_{\text{Ar, Tyr}}$), 6.74 (d, $\mathbf{H}_{\text{Ar, Tyr}}$), 4.63 (m, $C_{\alpha,\text{ Tyr}}\mathbf{H}$), 4.57 (t, $C_{\alpha,\text{ Phe}}\mathbf{H}$), 4.38 (m, $C_{\alpha,\text{ Leu}}\mathbf{H}$), 3.82 (m, $C_{\alpha,\text{ Gly}}\mathbf{H_2}$), 3.63 (s, C≡C**H**), 3.22 (dd, $C_{\beta,\text{ Tyr}}\mathbf{H}$), 3.08 (dd, $C_{\beta,\text{ Phe}}\mathbf{H}$), 3.03 (dd, $C_{\beta,\text{ Tyr}}\mathbf{H}$), 2.91 (dd, $C_{\beta,\text{ Phe}}\mathbf{H}$), 1.65 (m, $C_{\gamma,\text{ Leu}}\mathbf{H}$, $C_{\beta,\text{ Leu}}\mathbf{H_2}$), 0.94 (dd, $CH(C_{\delta,\text{ Leu}}\mathbf{H_3})_2$).

2: R_t = 16.4 min, MS (ESI^+, MeOH) : *m/z* 725 $[M - 6 \times CO + H]^+$, ^1H NMR (DMSO-d_6, 400.13 MHz): 9.08 (s, $O_{Tyr}\mathbf{H}$), 8.27 (t, $N_{Gly}\mathbf{H}$), 8.10 (m, 4H, $N_{\text{Gly, Phe, Tyr, Leu}}\mathbf{H}$,), 7.24 (m, $\mathbf{H}_{\text{Ar, Phe}}$), 7.01 (d, $\mathbf{H}_{\text{Ar, Tyr}}$), 7.00 (d, $CON\mathbf{H_2}$),6.61 (s, $Co_2C\mathbf{H}$), 6.58 (d, $\mathbf{H}_{\text{Ar ,Tyr}}$), 4.55 (m, $C_{\alpha,\text{ Tyr,Phe}}\mathbf{H}$), 4.20 (m, $C_{\alpha,\text{ Leu}}\mathbf{H}$), 3.74 (m, $C_{\alpha,\text{ Gly}}\mathbf{H_2}$), 3.01 – 2.79 (m, $C_{\beta,\text{ Tyr, Phe}}\mathbf{H}$), 1.56 (m, $C_{\gamma,\text{ Leu}}\mathbf{H}$) 1.47 (t, $C_{\beta,\text{ Leu}}\mathbf{H_2}$), 0.85 (dd, $CH(C_{\delta,\text{ Leu}}\mathbf{H_3})_2$).

3: R_t = 11.9 min, MS (ESI^-, MeOH): *m/z* 595 $[M-H]^-$, ^1H NMR (DMSO-d_6, 600.13 MHz): 9.13 (s, $O_{Tyr}\mathbf{H}$), 8.20 (t, $N_{Gly}\mathbf{H}$), 8.05 – 7.93 (m, $N_{Phe,Gly,Leu,,Tyr}\mathbf{H}$), 7.25 - 7.17 (m, $\mathbf{H}_{\text{Ar, Phe}}$), 7.07 (s, $CON\mathbf{H_2}$), 7.01 (d, 2H, $\mathbf{H}_{\text{Ar, Tyr}}$), 6.95 (s, 1H, $CON\mathbf{H_2}$), 6.63 (d, $\mathbf{H}_{\text{Ar, Tyr}}$), 4.51 (m, $C_{\alpha,\text{ Phe}}\mathbf{H}$), 4.39 (m, $C_{\alpha,\text{ Tyr}}\mathbf{H}$), 4.21 (m, $C_{\alpha,\text{ Leu}}\mathbf{H}$), 3.68 (m, $C_{\alpha,\text{ Gly}}\mathbf{H_2}$), 3.04 (dd, $C_{\beta,\text{ Phe}}\mathbf{H}$), 2.90 (dd, $C_{\beta,\text{ Tyr}}\mathbf{H}$), 2.79 (dd, $C_{\beta,\text{ Phe}}\mathbf{H}$), 2.63 (dd, $C_{\beta,\text{ Tyr}}\mathbf{H}$), 1.76 (s, $C_{Ac}\mathbf{H_3}$), 1.56 (m, $C_{\gamma,\text{ Leu}}\mathbf{H}$), 1.47 (m, $C_{\beta,\text{ Leu}}\mathbf{H_2}$), 0.85 (dd, $CH(C_{\delta,\text{ Leu}}\mathbf{H_3})_2$).

c) Cell culture and cytotoxicity test

The HeLa and HepG2 cell lines were obtained from ATCC. Cells were cultured in RPMI 1640 Medium (PAA Laboratories, 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 and in a humidified atmosphere with 5% CO_2. The cell lines were passaged weekly using 0.05% Trypsin with 0.02% EDTA (Gibco, Germany). The cell generation time under these conditions was determined and found to be between 20 – 24 h for both cell lines.

Two assays, the crystal violet assay (Bernhardt, G.; Reile, H.; Birnboeck, H.; Spruß, T.; Schönenberger, H. *J. Cancer Res. Clin. Oncol.* **1992,** *118*, 1, 35-43) and the resazurin assay (O'Brien, J.; Wilson, I.; Orton, T.; Pognan, F. *Eur. J. Biochem.* **2000,** *267*, 5421-5426, both Sigma) were performed on 96-well microtiterplates. 99 µL of a 40000 cells/ml suspension in culture medium were plated into each well and incubated for 24h at 37°C and 5% CO_2. By addition of 1 µL of a stock solution (100 mM, 50 mM and 10 mM) of the respective compound in methanol the desired concentrations (1 mM, 500 µM, and 100 µM) in 1% DMSO was reached. After 48h hour of incubation the cells first underwent the resazurin and subsequently the crystal violet assay. Cells were washed two times with colourless RPMI 1640 medium (PAA Laboratories, Germany) without Phenolred and without Fetal Calf Serum. Next, 90 µl of colourless RPMI 1640 and 10 µl of resazurin were added to each well. Absorbance was directly measured at 600 nm, and the measurement was repeated after two hours of incubation. Activity of mitochondrial dehydrogenases was determined by the decrease in absorbance. Next, the cell biomass was determined by a crystal violet assay. The medium was removed and cells were fixed with 4% paraformaldehyde in PBS. Cells were washed with PBS (phosphate buffered saline) and afterwards with 0.1% Triton-100 (Sigma) in PBS. Cells were then stained with a 0.04 % crystal violet solution and subsequently washed four times with distilled water. Crystal violet was extracted by 96% ethanol and the absorbance was determined at 570 nm. Values were corrected for the absorbance at the start of substance incubation.

Figure S1a) HPLC of Compound 1 (Alkyne-Enk-NH_2)

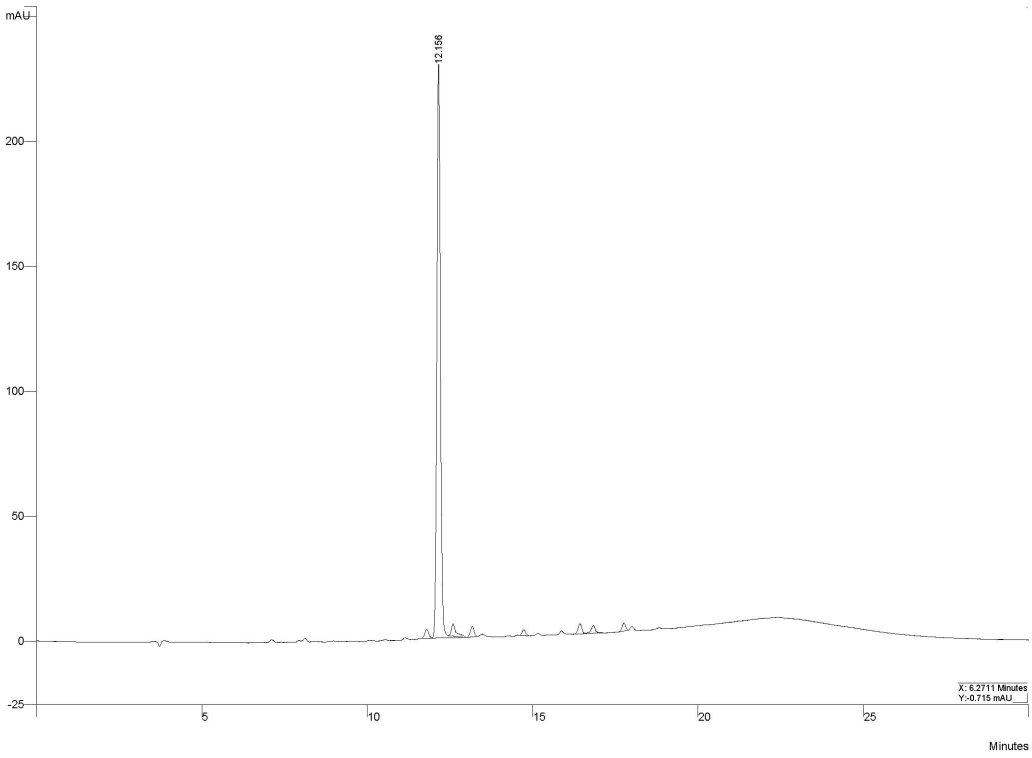

Figure S1b) ESI-MS of Compound 1 (Alkyne-Enk-NH_2)

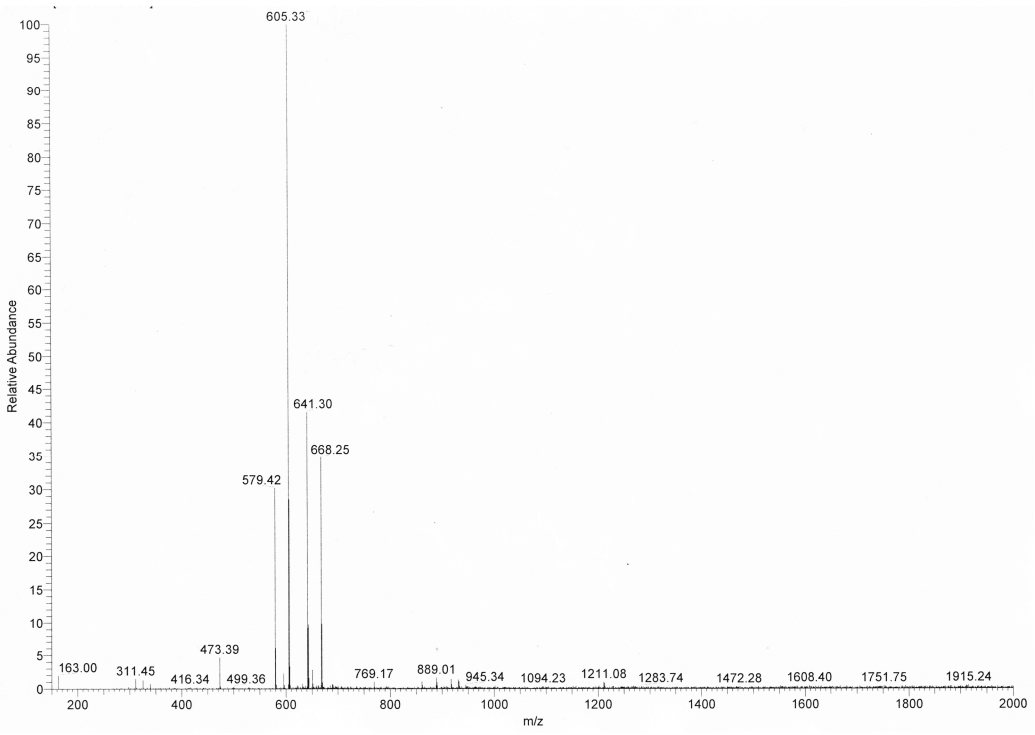

Figure S2a) HPLC of Compound 3 (Ac-Enk-NH_2)

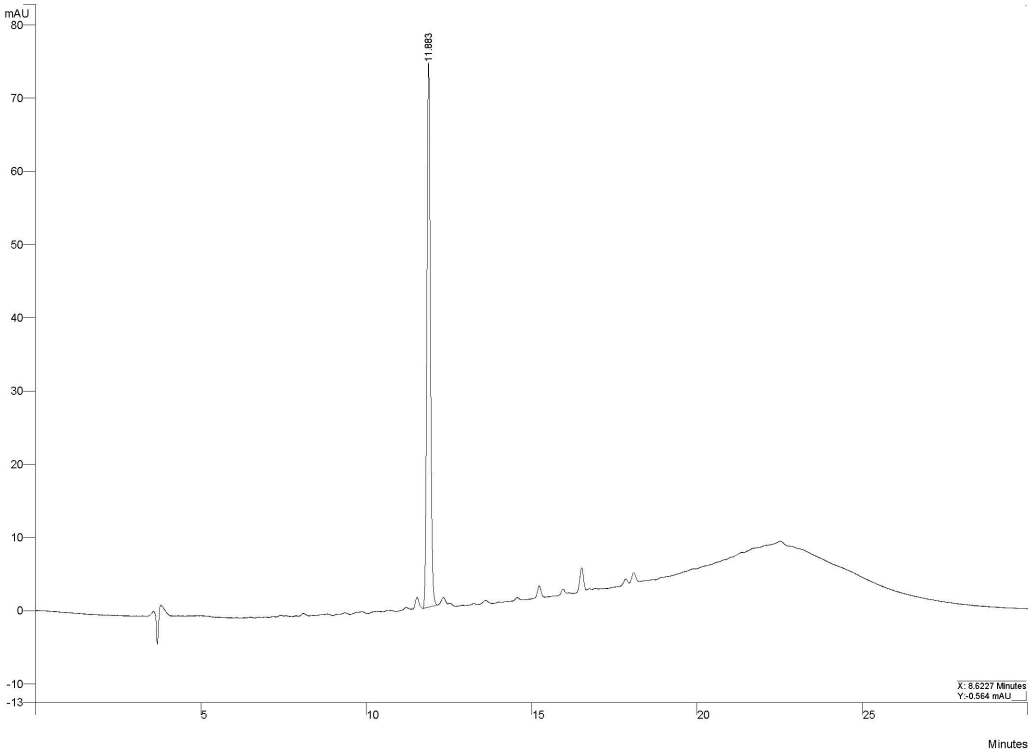

Figure S2b) ESI-MS of Compound 3 (Ac-Enk-NH_2)

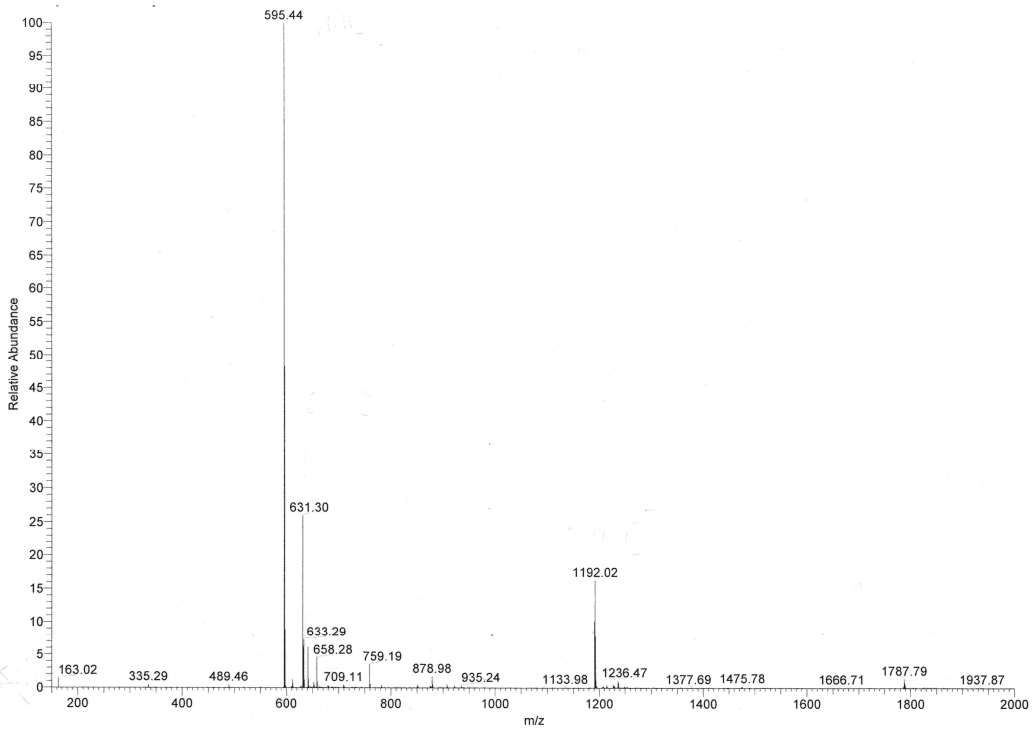

Figure S3a) HPLC of Compound 2 (Co-Enk-NH_2)

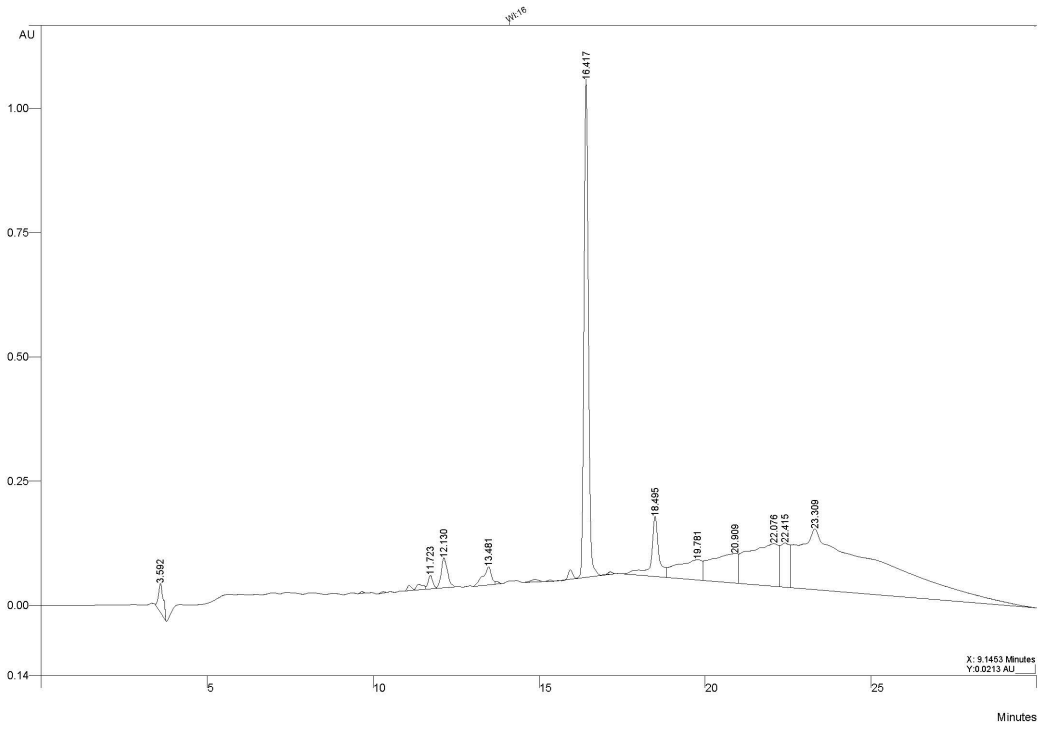

Figure S3b) ESI-MS of Compound 2 (Co-Enk-NH_2)

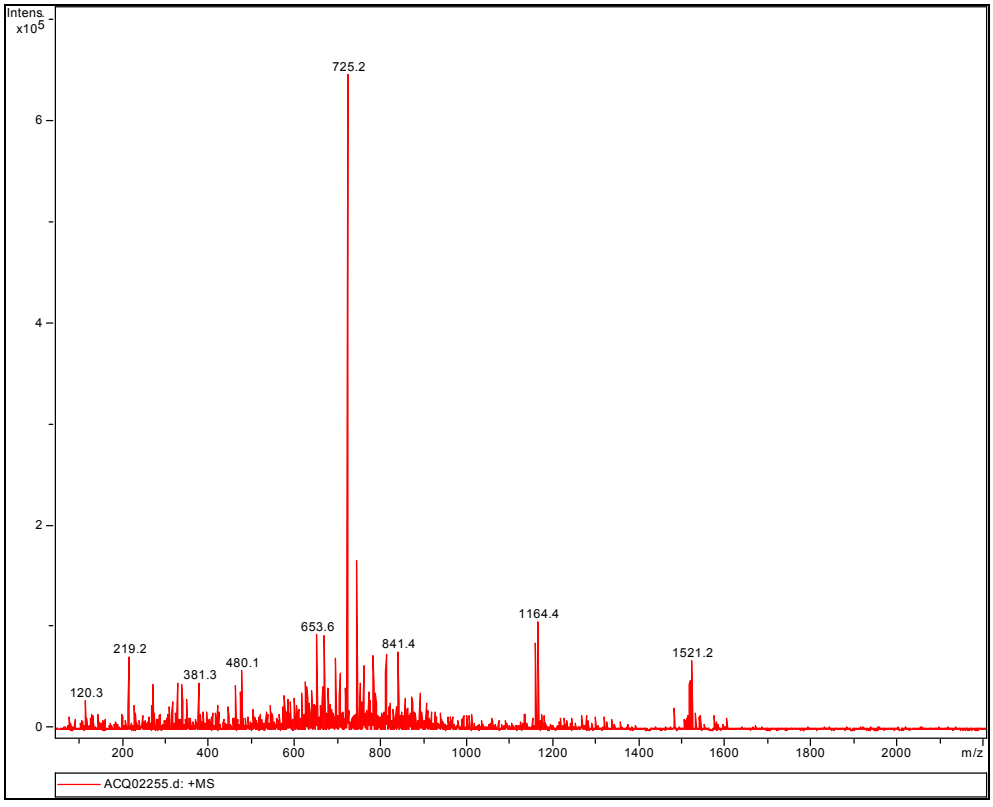

Figure S4a) HPLC of Compound 2 after 19 days. The compound was stored as a solid in an Eppendorf tube at -20°C.

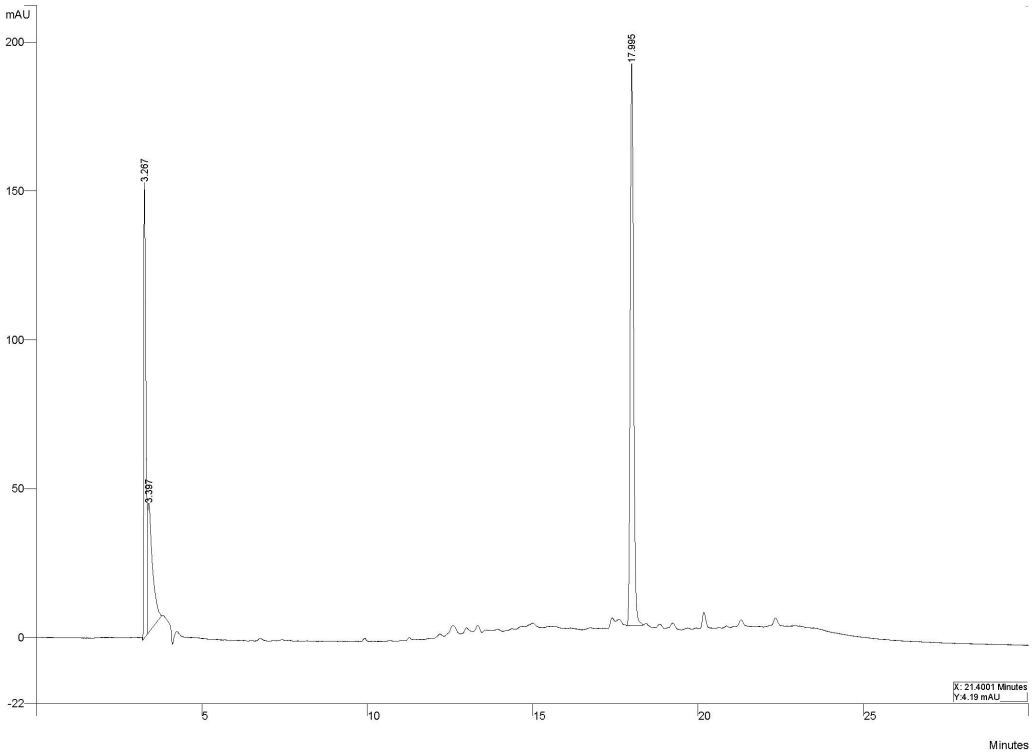

Figure S5a) IR spectrum of Compound 2 after 1 month (stored as KBr pellet on the bench without further protection)

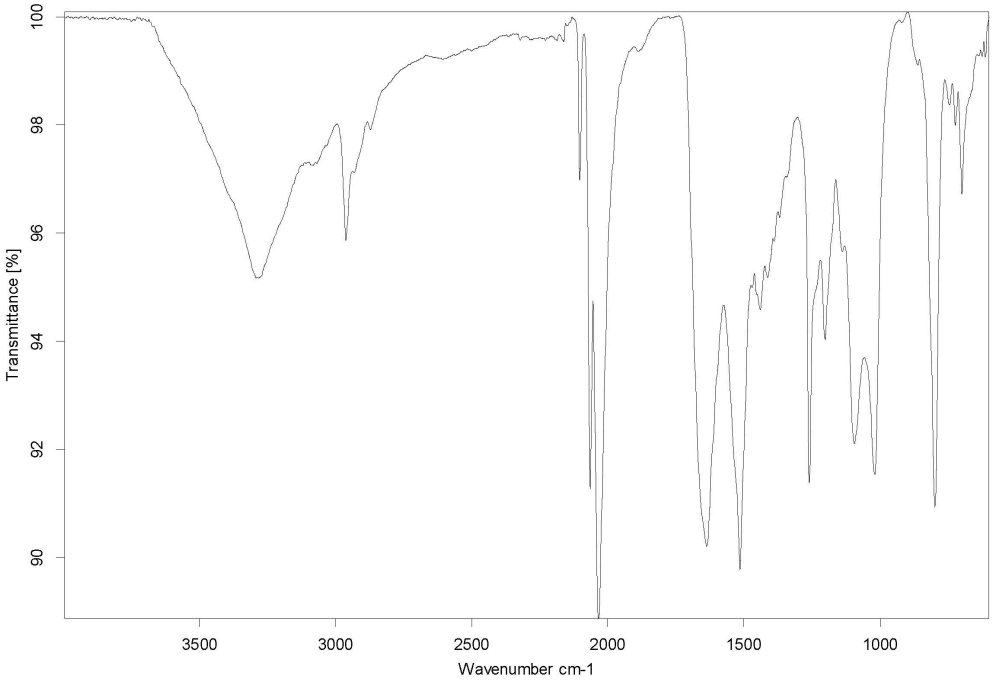

Figure S5b) IR spectrum of Compound 2 after 2 month (stored as KBr pellet on the bench without further protection)

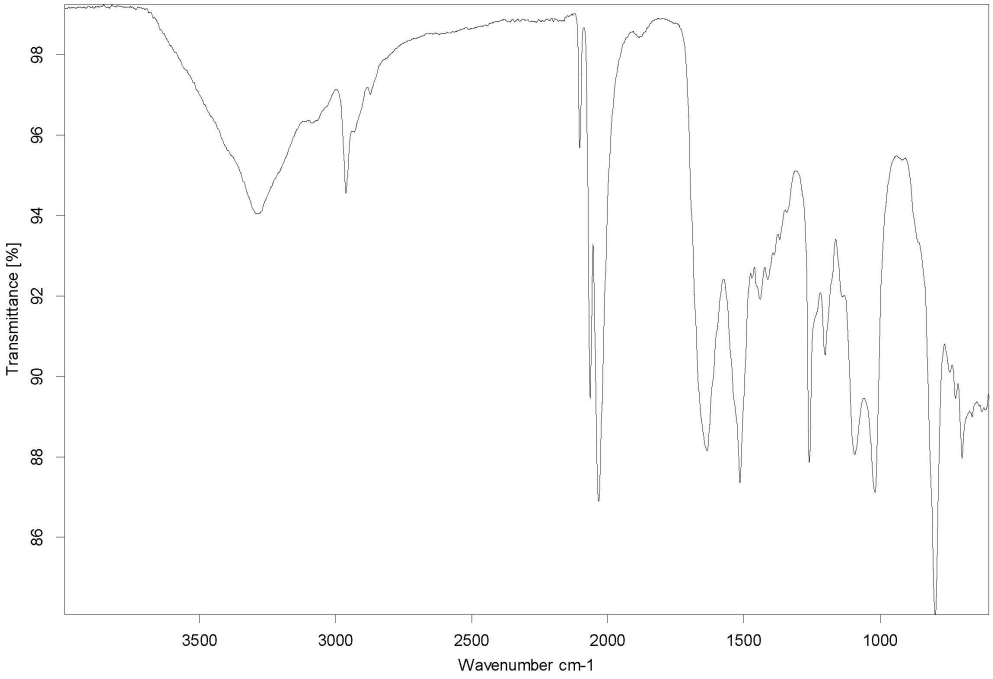

Figure S6a) IR spectrum of the $Co_2(CO)_6$-alkyne complex of the closely related peptide Phe-Gly-Gly-Phe-Leu-NH_2, recorded 7 days after preparation of the complex.

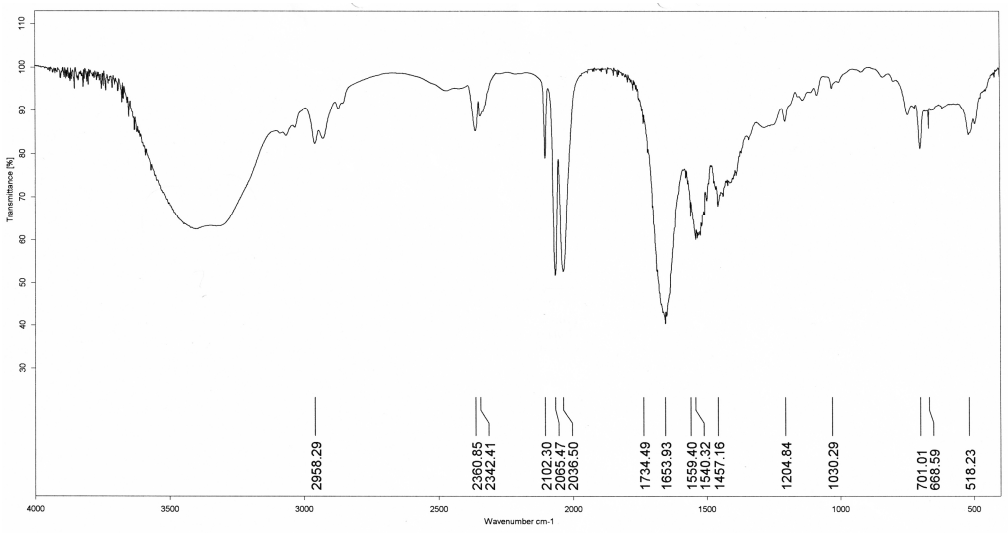

Figure S6b) IR spectrum of the $Co_2(CO)_6$-alkyne complex of the closely related peptide Phe-Gly-Gly-Phe-Leu-NH_2, recorded after 2 years (stored as KBr pellet on the bench without further protection).

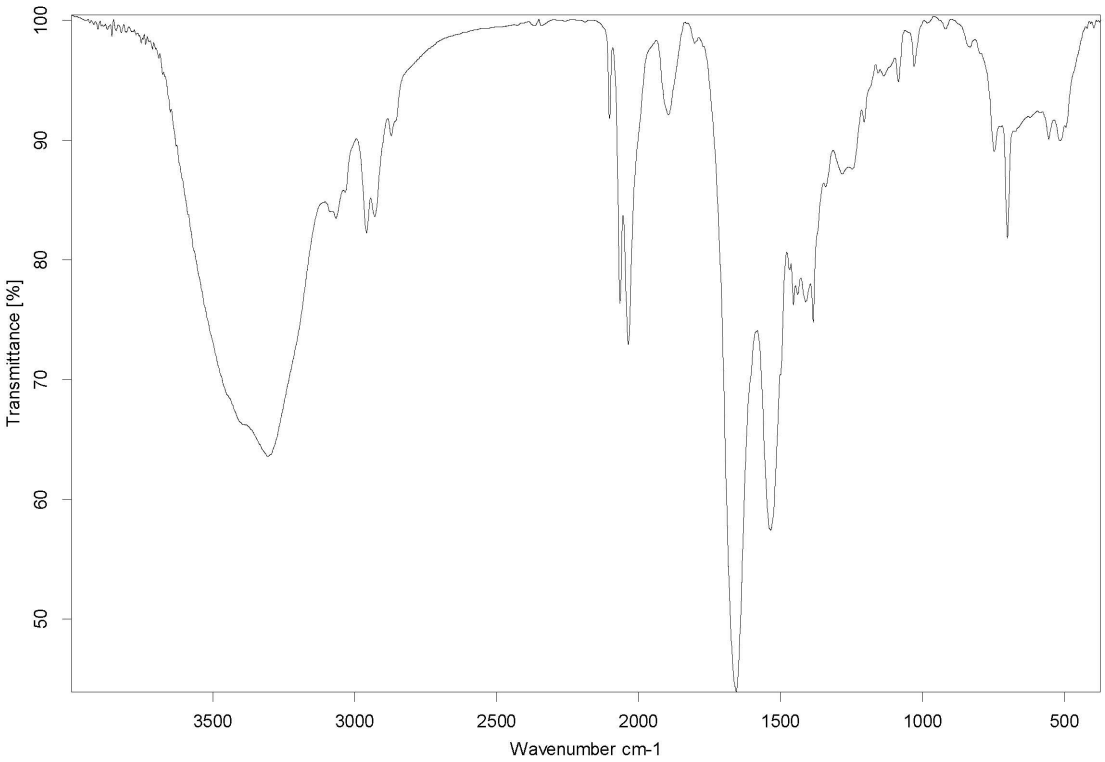

Figure S7:Cytotoxicity of $Co_2(CO)_8$. CV assay, conditions are the same as for Figure 2.