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Microwave-assisted solid-phase synthesis, cellular uptake, and cytotoxicity studies of cymantrene–peptide bioconjugates

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ABSTRACT

Five cymantrene ($CpMn(CO)_3$) bioconjugates of enkephalin (ENK), neurotensin (NT), pseudoneurotensin (pNT), NLS, and TAT were prepared by automated solid phase peptide synthesis (SPPS) under microwave irradiation in all steps, including peptide chain elongation, incorporation of the cymantrene moiety, final deprotection, and cleavage from the resin. The compounds were obtained in pure form with minimal work-up procedures and characterized by RP-HPLC, ESI-MS, and IR spectroscopy. They are efficiently internalized in HT-29 human colon cancer cells (0.22 to 0.48 nmol Mn/mg cell protein) and non-cytotoxic at up to 100 μM. With the carbonyl C=O bands clearly detectable even in the presence of an octapeptide chain as in the cymantrene–NLS conjugate, these compounds will be useful as IR spectroscopic probes in peptide biodistribution studies.

1. Introduction

In bioorganometallic chemistry, there is an ongoing interest in the labelling of physiologically active molecules with metal complexes for applications in the diagnosis and therapy of human diseases [1]. The functionalization of peptides is especially promising since they have a wide array of biological targets and can promote cellular uptake of attached "cargo" either via specific receptors or by passive diffusion across the membrane [2–5]. In addition, different amino acid sequences of considerable length can now easily be prepared with automated peptide synthesizers in a matter of hours, allowing access to a large chemical space in relatively short time. It would thus be particularly interesting if metal-based markers could also directly be attached to such carrier peptides during the automated solid-phase synthesis protocol with minimal side reactions and purification steps. While redox-active sandwich complexes have been quite frequently used in the labelling of peptides [6–14], other functional groups have received somewhat less attention [15–24]. In addition, most of these functionalities were introduced by manual coupling. We thus became interested in cymantrene ($CpMn(CO)_3$) as an easy-to-functionalize and robust IR spectroscopic marker [25–33] and show here that microwave-assisted solid-phase synthesis allows one to prepare different cymantrene–peptide bioconjugates of increasing complexity in good yield and purity. [Leu^5]-enkephalin (ENK) was chosen as a simple pentapeptide with few charged amino acid residues to start with and validate the solid-phase peptide synthesis (SPPS) method. It is a natural ligand for opiate receptors in the central nervous system (CNS) and has been used in model studies before [19,34–36]. Neurotensin (NT) and pseudoneurotensin (pNT) also act as neurotransmitters and neuromodulators in the CNS and additionally exhibit an endocrine effect in the periphery of the body [37–39]. With one tyrosine and two arginine or lysine residues, respectively, this hexapeptide is of moderate length and has a limited number of charged residues. The nuclear localization sequence (NLS) facilitates transfer across the membrane separating the cell nucleus from the cytosol and has a high number of charged (Lys, Arg) amino acids, six out of eight in the octapeptide sequence chosen from the SV-40 T antigen in this study [40,41]. The HIV transactivator of transcription (TAT) protein still contains a larger number of charged amino acids and is used to challenge the synthetic methodology. With 12 amino acids from the TAT48-60 sequence, it is the longest peptide studied and expected to enable translocation through the plasma membrane and into the nucleus [42]. In biodistribution studies of metal carbonyl–peptide conjugates, the metal itself can also serve as an intrinsic marker in addition to the high intensity IR bands of the CO ligands. Atomic absorption spectroscopy was therefore used to determine cell-associated manganese levels in HT-29 human colon cancer cells. Finally, a spectroscopic probe should not alter the system under study. Thus, the cytotoxicity of all cymantrene–peptide bioconjugates was investigated with a cell viability assay.

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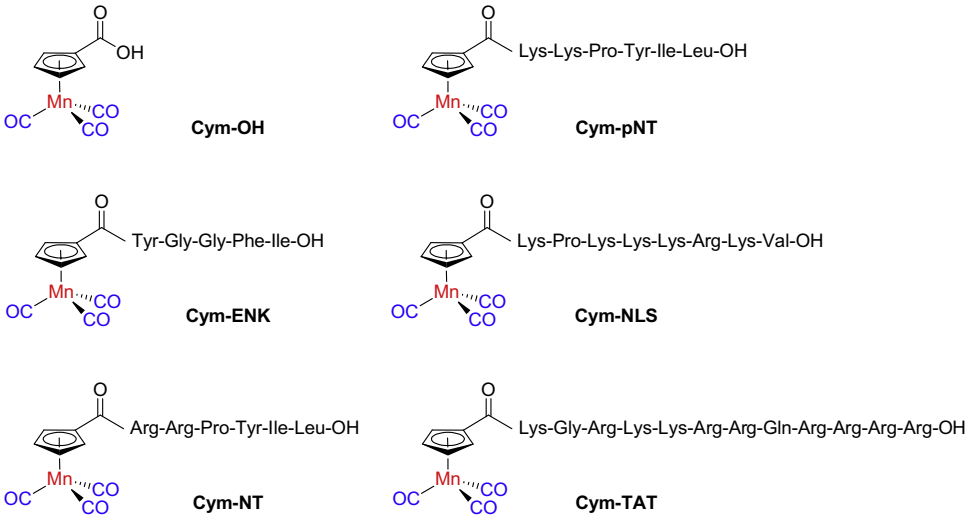
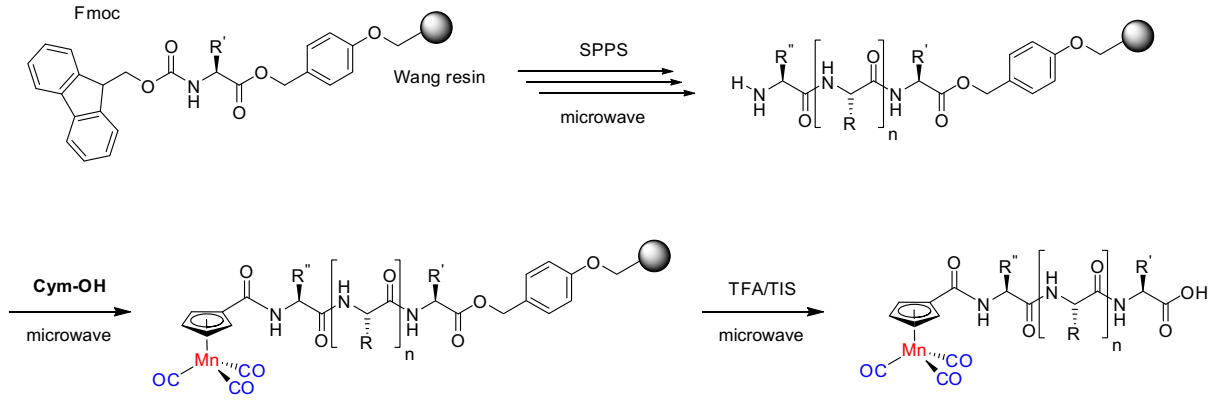
2. Results and discussion

2.1. Precursor and solid-phase synthesis

For the direct attachment of the cymantrene moiety to the *N*-terminus of a peptide, cymantrene carboxylic acid **Cym-OH** (Scheme 1) was prepared in a two-step reaction sequence from cymantrene following the procedure of Biehl et al. [43]. Five different peptides of increasing length were then assembled by standard procedures using the Fmoc protective group strategy on a Wang resin in an automated microwave-assisted synthesizer (Scheme 2) [44,45]. Both coupling and N-terminal deprotection steps were done under microwave irradiation. After attachment of the final amino acid, the Fmoc group was removed by treatment with 20% piperidine in dimethylformamide. Still under full control of the synthesizer and with microwave irradiation, **Cym-OH** was than coupled to the resin-bound peptide using TBTU as the activating agent and an excess of diisopropylethylamine as the base. Cleavage of the cymantrene-functionalized peptide from the solid support and deprotection of the amino acid side-chains was performed in one step with 95% trifluoroacetic acid containing 2.5% water and 2.5% triisopropylsilane to quench reactive intermediates, also in the synthesizer. Only then, the dissolved product was removed from the instrument, the solvent reduced in vacuo to 1–2 ml and the organometallic peptide conjugate precipitated with ice-cold diethylether. After two more cycles of centrifugation and precipitation, the combined solid material was dried in the air. After preparative HPLC, the main fraction of cymantrene–peptide conjugate was dissolved in a mixture of water/acetonitrile, lyophilized, and checked again with HPLC. For all peptides, the analytical RP-HPLC trace showed only one major peak as presented in Fig. 1 for **Cym-NLS** as an example. ESI mass spectrometry gave $[M+2H]^{2+}$ as the base peak together with the signals of $[M+H]^{+}$ and $[M+3H]^{3+}$ for all compounds (see Fig. 1 for the spectrum of **Cym-NLS** as an example) except for **Cym-ENK**, which is best measured in the negative mode showing the $[M-H]^{-}$ signal due to the acidity of the tyrosine. The IR band positions for the metal carbonyl C=O vibrations vary very little and are found at 1938 ± 1 and 2027 ± 1 cm^{-1}, respectively, with the exception of **Cym-ENK**, where they are shifted to slightly lower energies (1932 and 2024 cm^{-1}), indicating very little influence of the peptide on the electronic properties of the cymantrene moiety. Even in the presence of an octapeptide chain as in **Cym-NLS**, the metal carbonyl bands are clearly visible and distinct from the strong amide bands in the 1610–1670 (amide I) and 1530 (amide II) cm^{-1} region (Fig. 2).



Scheme 1. Starting material **Cym-OH** and cymantrene–peptide bioconjugates **Cym-ENK**, **Cym-NT**, **Cym-pNT**, **Cym-NLS**, and **Cym-TAT** studied in this work.



Scheme 2. General procedure of fully automated and microwave-assisted solid-phase synthesis of cymantrene–peptide bioconjugates.

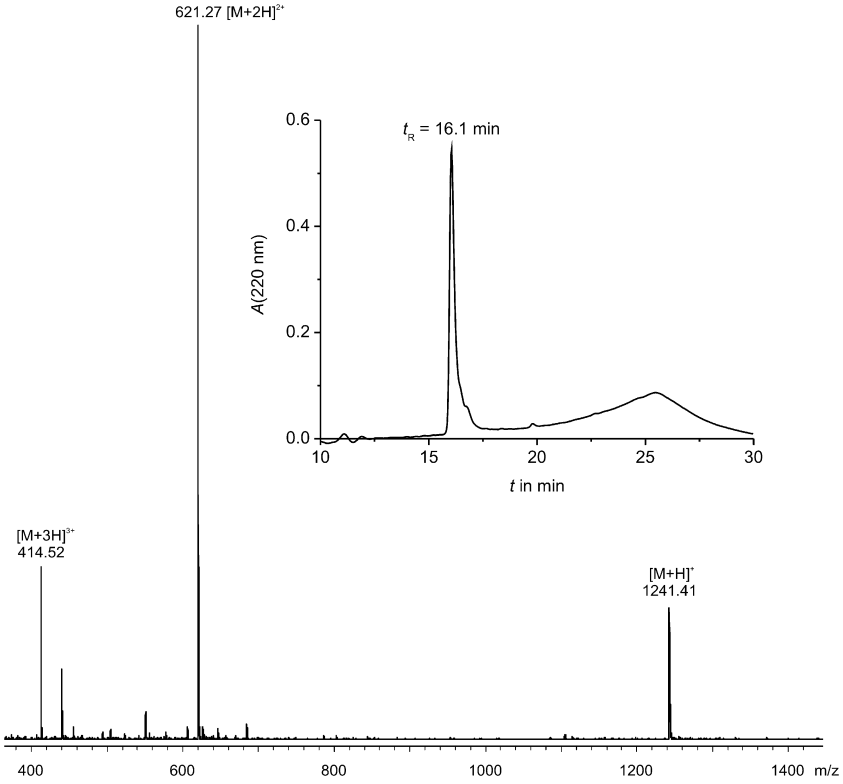


Fig. 1. Positive mode ESI mass spectrum of the **Cym-NLS** bioconjugate in methanol. The inset shows the RP-HPLC trace of the compound.

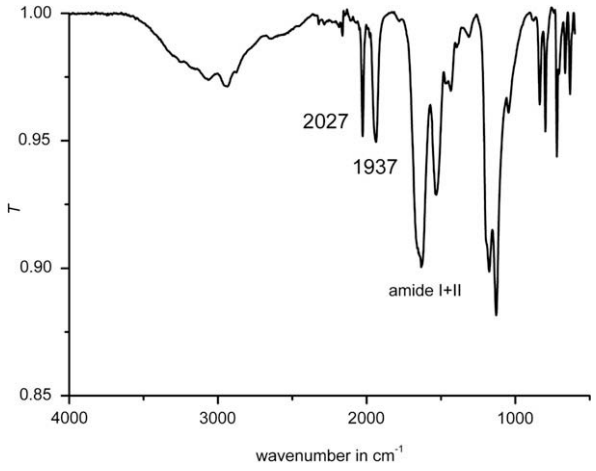


Fig. 2. ATR-IR spectrum of the **Cym-NLS** bioconjugate. The two C═O bands of the $CpMn(CO)_3$ moiety at 1937 and 2027 cm^{-1} are clearly distinct from the signals of the peptide, most notably the intense amide I + II bands.

2.2. Cellular uptake studies

Cellular uptake into HT-29 human colon cancer cells after 6 h of exposure was studied using a recently established assay based on graphite-furnace atomic absorption spectroscopy [46]. With values between 0.22 to 0.35 nmol Mn/mg of cell protein, the uptake of the conjugates containing the ENK, NT, NLS, and TAT sequences was almost independent of the nature of the peptide chain (see Table 1). Somewhat distinct is, however, the **Cym-pNT** conjugate (upake: 0.48 nmol Mn/mg cell protein), which shows approximately twice as high intracellular accumulation as the closely related **Cym-NT** (0.22 nmol Mn/mg cell protein). The structures of **Cym-pNT** and **Cym-NT** differ by the substitution of two arginine residues of the NT sequence with lysines in the pNT sequence. The consequence of this change in the amino acid sequence is a lower basicity (and thereby higher lipophilicity) of pNT compared to NT, which is protonated at both arginine side chains under physiological conditions. Similar conclusions are difficult to draw for the remaining cymantrene–peptide conjugates as their peptide sequences differ more strongly from each other and the uptake of peptide derivatives is also dependent on the presence of various specific cellular transporters. As 1.0 mg of protein from HT-29 cells corresponds to a cell volume of 5.1 μL, the molar intracellular concentration of 1.0 nmol Mn per 1.0 mg protein is 196 μM [47]. Based on this estimation of the molar cellular Mn concentration and after dividing by the exposure concentration of 50 μM, the cellular accumulation of **Cym-pNT** was found to be 1.9. That is, the intracellular concentration exceeds the extracellular one approximately twofold. For the other four peptides, the mean cellular accumulation is between 0.9 and 1.4. Thus, the concentration of these bioconjugates inside the cells essentially equals that of the medium.

Table 1
Cellular uptake into HT-29 colon carcinoma cells after 6 h of exposure to 50 μM of compounds as determined by GF-AAS

Compound	Cellular uptake nmol Mn/mg cell protein
Cym-ENK	0.24 ± 0.11
Cym-NT	0.22 ± 0.05
Cym-pNT	0.48 ± 0.11
Cym-NLS	0.24 ± 0.09
Cym-TAT	0.35 ± 0.11

2.3. Cytotoxicity

The influence of all five cymantrene–peptide conjugates on the viability of HT-29 human colon cancer cells was studied with the crystal violet (CV) assay. In this method, the UV–Vis absorption of CV is directly proportional to the amount of cell biomass present. In all cases, no reduction of cell biomass relative to the controls was found at up to 100 μM. Thus, the compounds can be considered as non-cytotoxic, as required for applications in biological labelling.

3. Conclusion

Cymantrene is a robust organometallic biomarker which can be introduced to peptides using a fully automated solid-phase synthesis protocol under microwave irradiation in all steps to give bioconjugates in good yield and purity. The organometallic $CpMn(CO)_3$ group even survives cleavage from the resin with 95% trifluoroacetic acid. The peptide conjugates thus obtained are efficiently internalized in HT-29 human colon cancer cells and non-cytotoxic. With the metal carbonyl C═O bands clearly detectable even in the presence of a long peptide chain, these compounds will be useful as IR spectroscopic probes in peptide biodistribution studies. Work along these lines is in progress.

4. Experimental

4.1. General remarks

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. No special precautions were undertaken to protect the compounds from light or oxygen, but direct exposure to sunlight was avoided when possible and samples were kept in the dark in a refrigerator for long-term storage. Mass spectra were measured on a Bruker Esquire 6000 (ESI) instrument. Only characteristic fragments are reported with the MS peak positions given for the most intense line of the isotope distribution observed. The solvent flow rate for the ESI measurements was 4 μl 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. IR spectra were recorded on pure solid samples using a Bruker Tensor 27 IR spectrometer equipped with a Pike MIRacle Micro ATR accessory (ATR = attenuated total reflectance). The analytical and preparative HPLC measurements were performed on a Varian ProStar instrument using a Dynamax RP analytical column (C18 microsorb 60 Å, diameter 4.5 mm, 250 mm length) and preparative column (C18 microsorb 60 Å, diameter 10 mm, 250 mm length), both 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 (4 mL/min, respectively).

4.2. Synthetic procedures

Cymantrene carboxylic acid was prepared in two steps from cymantrene following the procedure of Biehl et al. [43]. The peptides were prepared using 9-fluorenylmethoxycarbonyl (Fmoc) strategy on a CEM Liberty microwave peptide synthesizer on a 0.1 mmol scale. The side-chain protective groups Pfb (2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl), Trt (trityl), Boc (*t*-butoxycarbonyl), and *t*Bu ether were used for arginine, glutamine, lysine, and tyrosine, respectively. Wang resin served as the solid support. It was preloaded with the first protected amino acid at a degree of functionalization of 0.73 mmol per gram of resin. The coupling was performed in dimethylformamide (DMF) and the peptide chain assembled under computer control of the synthesizer. The synthesis cycle was composed of Fmoc-deprotection and coupling, both under microwave irradiation. The deprotection was performed with 20% piperidine in DMF while the coupling mixture contained Fmoc-protected amino acid (0.2 M in DMF), the activating agent TBTU (*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate, 0.45 M in DMF) and an excess of diisopropylethylamine (DIPEA) as the base. In the last coupling step, the cymantrene carboxylic acid (0.2 M in DMF) was used in place of the amino acid using an otherwise unchanged protocol. The final cleavage of the cymantrene-functionalized peptide from the solid support and the deprotection of the side chains was performed with 95% trifluoroacetic acid (TFA) containing 2.5% water and 2.5% triisopropylsilane (TIS) to quench reactive intermediates under microwave irradiation. The C-terminal end of the peptide is obtained as the free carboxylic acid in this case. The solvent was reduced in vacuo to 1–2 ml and then ice-cold diethylether added to precipitate the peptide. After centrifugation, the solvent was decanted and treated again with ice-cold diethylether to obtain more precipitate. After two more such cycles, the combined solid material was dried in the air and analyzed with HPLC and ESI-MS for purity. The peptides were dissolved in water/acetonitrile and purified by preparative HPLC. The analytical pure peptides were then collected as solids after lyophilization. **Cym-ENK**: ESI-MS(CH_3OH, neg. mode): 784.13 $[M-H]^-$; IR $\tilde{\nu}$ (CO): 2024, 1932 cm^{-1}; t_R = 15.7 min. **Cym-NT**: ESI-MS(CH_3OH, pos. mode): 1047.30 $[M+H]^+$, 524.18 $[M+2H]^{2+}$; IR $\tilde{\nu}$ (CO) 2026, 1939 cm^{-1}; t_R = 18.4 min. **Cym-pNT**: ESI-MS(CH_3OH, pos. mode): 991.30 $[M+H]^+$, 496.17 $[M+2H]^{2+}$; IR $\tilde{\nu}$ (CO) 2026, 1937 cm^{-1}; t_R = 15.4 min. **Cym-NLS**: ESI-MS(CH_3OH, pos. mode): 1241.43 $[M+H]^+$, 621.28 $[M+2H]^{2+}$, 414.53 $[M+3H]^{3+}$; IR $\tilde{\nu}$ (CO) 2027, 1937 cm^{-1}; t_R = 16.1 min. **Cym-TAT**: ESI-MS(CH_3OH, pos. mode): 785.31 $[M+2H]^{2+}$, 523.90 $[M+3H]^{3+}$; IR $\tilde{\nu}$ (CO) 2026, 1938 cm^{-1}; t_R = 11.4 min.

4.3. Cellular uptake studies

Cellular uptake studies were performed according to a previously described method with minor modifications [46,47]. In short, HT-29 cells were grown to at least 70% confluency in 175 cm^2 cell culture flasks. Stock solutions of the cymantrene–peptide conjugates in dimethylsulfoxide (DMSO) or water were freshly prepared and diluted with cell culture medium to the desired concentrations (final DMSO concentration: 0.1% v/v, final compound concentration: 50 μM). The medium of the cell culture flasks was replaced with 10 mL of medium solutions containing the compound and the flasks were incubated at 37 °C/5% CO_2 for 6 h. The cell pellets were isolated, resuspended in 1 mL doubly-distilled water, lysed with a sonotrode and appropriately diluted using doubly-distilled water. An aliquot was removed for protein quantification by the Bradford method. Prior to manganese quantification by GF-AAS 20 μL Triton X-100 (1%) and nitric acid (13%) were added to each 200 μL of the cell suspension. Details of the GF-AAS method have already been reported [46]. Cellular uptake is expressed as nmol manganese per mg cell protein. The manganese background concentration of blank pellets was subtracted from the probes. Data were obtained from 2 to 3 independent experiments.

4.4. Cytotoxicity measurements

The antiproliferative effects of the compounds were determined following an established procedure [47]. In short, cells were suspended in cell culture medium (2850 cells/mL), and 100 μL ali-

quots thereof were plated in 96 well plates and incubated at 37 °C/5% CO_2 for 48 h. Stock solutions of the compounds in dimethylsulfoxide were freshly prepared and diluted with cell culture medium to the desired concentrations (final dimethylsulfoxide concentration: 0.1% v/v). The medium in the plates was replaced by medium containing the compounds in graded concentrations (six replicates). After further incubation for 72 h, cell biomass was determined by crystal violet staining and the IC_{50} values were determined as those concentrations causing 50% inhibition of cell proliferation, when observed. Results were calculated from two to three independent experiments.

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