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Labelling of [Leu^5]-enkephalin with organometallic Mo complexes by solid-phase synthesis†‡

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The neuropeptide leucine-enkephalin ([Leu^5]-enkephalin) has been covalently labelled with the two organometallic Mo carbonyl complexes Mo(R-His)(allyl)(CO)$_2$ (His = N_δ,*N*,*O*-L-histidinate) and Mo(R-bpa)(CO)$_3$ (bpa = bis(2-picolyl)amine) on the resin as the last step of a solid-phase synthesis scheme and in solution.

Inorganic compounds possess properties which are not present in biomolecules such as proteins or DNA. Therefore, the labelling of biomolecules with inorganic complexes is an active area of research.[1] Organometallic complexes have long been neglected for this purpose, mainly because they are conceived to be too unstable in an aqueous, aerobic environment. Recently, several examples for the labelling of DNA and PNA (a structural mimic of DNA) with ferrocene were reported.[2–5] Jaouen *et al*. have utilized a variety of organometallic compounds for the labelling of hormones,[6] drugs (*e.g*. barbiturates) and proteins.[7,8] Compared to other classes of biomolecules, the labelling of small oligopeptides has hardly been mentioned.[9–15] In this work, we present two approaches for the labelling of oligopeptides with organometallic Mo carbonyl complexes. The neuropeptide [Leu^5]-enkephalin (Enk; H-Tyr-Gly-Gly-Phe-Leu-OH), which is a natural ligand to the opiate receptor, was chosen as the target for labelling.

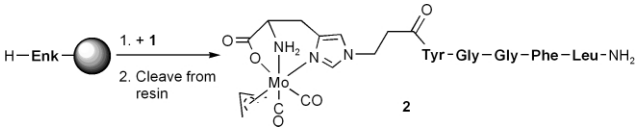
We have published synthetic and spectroscopic work on Mo carbonyl complexes of the type Mo(N_ε-R-His)(η^3-allyl)(CO)$_2$ (His = N_δ, *N*,*O*-L-histidinate) and Mo(R-bpa)(CO)$_3$ (bpa = bis(2-picolyl)amine).[16–19] These compounds are particularly suited for applications in bio-organometallic chemistry by virtue of their physicochemical properties: highly sensitive IR detection of the strong metal carbonyl CO stretching vibrations, excellent electrochemical properties and very good stability of the one-electron oxidized paramagnetic species. In addition, they were found to be exceptionally stable in air and water. The complexes Mo(N_ε-C_2H_4-CO_2Me-His)(allyl)(CO)$_2$,[17,18] however, were found to decompose in acidic media. In order to make this complex amendable to solid-phase synthesis, the following synthesis scheme was devised (Scheme 1).

Enkephalin was synthesized by standard Fmoc solid-phase methods on NovaSyn TGA resin with an HMBA linker.[20] Tyr was introduced as its *O*(2-Cl-Trt) protected derivative, and the 2-Cl-Trt group was removed (5% TFA and 5% TIS in CH_2Cl_2). Subsequently, the Tyr-Fmoc group was removed by piperidine and the metal complex Mo(N_ε-$C_2H_4CO_2$H-His)(allyl)(CO)$_2$ **1**[21] was coupled to the resin-bound, fully deprotected Enk after activation with TBTU. The Mo(His)–Enk bioconjugate **2** was cleaved from the resin by treatment with saturated NH_3 solution in MeOH and purified by preparative HPLC to yield about 100 mg (*ca*. 70% based on the resin used) of **2** in highly pure form.

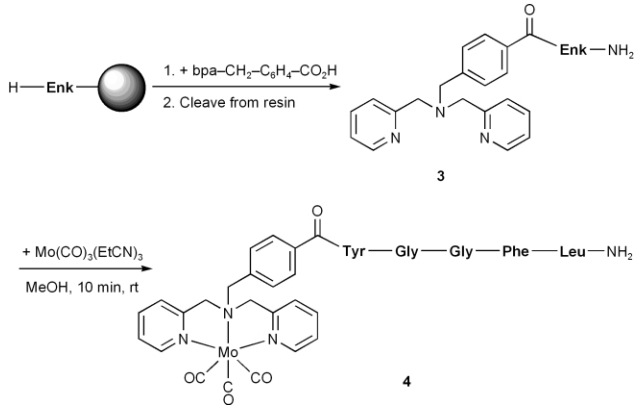
The aforementioned procedure of attaching a metal complex to the peptide on the solid support is certainly the most elegant way. However, a situation may arise when this is not possible or desirable, *e.g*. with a radioactive metal isotope complex. In such cases, an innocent anchoring group can be attached to the peptide during solid-phase synthesis. The ligand–peptide conjugate is then cleaved from the resin, purified and the metal label is only added in solution immediately prior to use of the bioconjugate. In the following, we provide an example for this procedure using the Mo(CO)$_3$ fragment and bis(2-picolyl)amine (bpa) as a ligand (Scheme 2).

Enkephalin was prepared by solid-phase synthesis and deprotected as described above. Subsequently, the resin was reacted with a five-fold excess of *N*-(*p*-carboxybenzyl)bis(2-picolyl)amine (bpa-$CH_2C_6H_4CO_2$H)[19] under TBTU activation. After cleavage from the resin and HPLC purification, *ca*. 130 mg (80%) of the pure bpa–Enk derivative **3** were obtained. All analytical data were consistent with the proposed constitution (see ESI†). A high-resolution ESI-MS on the [**3** + Li]$^+$ peak yielded an exact mass of 876.4380, in excellent agreement with the calculated mass (876.4384). After reacting **3** with a stoichiometric amount of Mo(CO)$_3$(EtCN)$_3$ in MeOH for 10 min at room temperature an orange precipitate formed. The solution was evaporated to dryness to avoid any loss of compound, affording the Mo(bpa)–Enk conjugate **4** quantitatively in pure form.

Both Mo-enkephalin conjugate complexes are yellow. A successful labelling to yield resin-bound **2** is immediately indicated by the yellow colour of the resin which does not



Scheme 1 Solid-phase synthesis of Mo(His)–Enk conjugate **2**. The Mo(His)(allyl)(CO)$_2$ complex is attached on the resin, and the *whole* conjugate is cleaved in the last synthetic step.



Scheme 2 Synthesis of the Mo(bpa)–Enk conjugate **4**. The bpa ligand is attached to the peptide on the solid support and the Mo(CO)$_3$ fragment is added to this anchoring group *after* cleavage from the resin.

† Electronic supplementary information (ESI) available: full analytical data for **2**–**4**. See http://www.rsc.org/suppdata/cc/b2/b203128k/

‡ *Abbreviations*: HMBA = hydroxymethylbenzoic acid; 2-Cl-Trt = 2-chlorotrityl; TFA = trifluoroacetic acid; TIS = triisopropylsilane; TBTU = *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; Enk = leucine-enkephalin, [Leu^5]-enkephalin; HOBt = hydroxybenzotriazole.

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Fig. 1 Photograph of the resin with (left) or without (right) the Mo complex **1** attached. The success of the labelling can be visually verified by the yellow colour of the resin.

disappear upon repeated washing steps (Fig. 1). Characteristic low-wavelength absorptions of the metal complexes also facilitate HPLC identification and purification of the conjugates. Evidence for the proposed constitution of **2** and **4** was obtained from ESI-positive MS. Clusters of peaks with the correct isotope patterns were observed at m/z = 958 $[M + H]^+$ and m/z = 980 $[M + Na]^+$ (for **2**) and m/z = 1052 $[M + H]^+$ and m/z = 1074 $[M + Na]^+$ (for **4**). In addition, a high-resolution ESI-MS could be recorded for **2**, yielding an exact mass for the $[M + Na]^+$ peak of m/z = 980.2826, in good agreement with the calculated mass (980.2817).

Both conjugates are also unambiguously identified by ^{1}H and ^{13}C NMR spectroscopy (see ESI†). All signals from the Enk were readily assigned by 2D NMR methods. An exchangeable ^{1}H NMR signal at 9.2 ppm for the phenolic proton verifies complete Tyr-OH deprotection. The presence of metal carbonyl ligands is confirmed by ^{13}C NMR signals at 230.2 and 228.3 ppm (**2**), and at 231.2 and 230.3 ppm (intensity 2 : 1, **4**). Finally, the KBr IR spectra of the conjugates show stretching vibrations of the Mo carbonyl groups at 1830 and 1930 cm^{-1} (**2**) or at 1761, 1787 and 1896 cm^{-1} (**4**), respectively (Fig. 2). These wavenumbers are almost identical to those of the free metal complexes.[18,19] The bioconjugate **2** exists as a mixture of two regioisomers in a 55 : 45 ratio in DMSO as demonstrated previously for the parent complex Mo(N_ε-$C_2H_4CO_2Me$)(allyl)$(CO)_2$.[17,18]

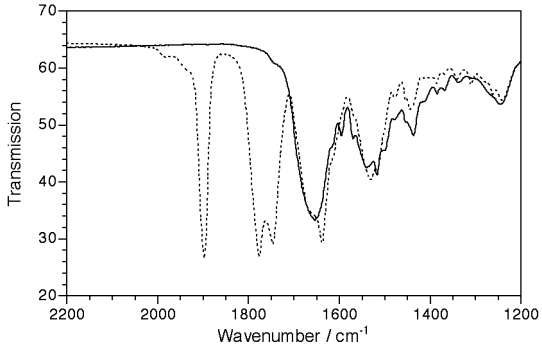

Fig. 2 IR spectra (KBr) of bpa-Enk **3** (solid line) and Mo$(CO)_3$(bpa-Enk) **4** (dotted line). Strong metal carbonyl CO stretching vibrations occur around 1700–1900 cm^{-1} in an otherwise blank region of the spectrum.

Electrochemical detection is a further property that metal complexes may add to the otherwise unactive peptides. Both conjugates could be reversibly one-electron oxidized in DMF at +20 mV *vs.* Fc/Fc$^+$ (**2**) and −220 mV *vs.* Fc/Fc$^+$ (**4**), respectively. In earlier work, we have confirmed that these oxidations are indeed metal centred. The stability of the one-electron oxidized metal complexes is high and has permitted detailed spectroscopic studies of those paramagnetic organometallic complexes.[18,19]

There are a number of applications for organometallic bioconjugates. Jaouen and coworkers have developed an immunoassay based on IR spectroscopy (*C*arbonyl *M*etallo *I*mmuno *A*ssay, CMIA).[1,22,23] Radio-imaging with ^{99m}Tc-labelled antibodies was reported by Alberto and coworkers using the new Tc$(CO)_3$ label.[1,24] Last year, a DNA mismatch analysis was reported based on electrochemical detection of ferrocene-labelled oligonucleotides.[4] All of these new developments rely on analytical detection of a property that is unique to the (organometallic) complex in the conjugates. The selective labelling of oligopeptides is a synthetic challenge for applications in medicine and molecular biotechnology. In this work, we report new peptide bioconjugates with organometallic Mo carbonyl complexes. The conjugates were prepared in excellent yield and purity by two different solid phase synthesis strategies. Their physiological properties and possible application as markers for neurochemistry are currently being investigated in the Heidelberg group.

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