Alternative Solid-Phase Strategies for the Efficient Labelling of Peptides with (Bathophenanthroline)ruthenium(II) Complexes

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To increase the flexibility for the insertion of highly sensitive and robust [Ru^{II}(bathophenanthroline)] complexes into peptides, we have evaluated three different solid-phase strategies (bathophenanthroline = 4,7-diphenyl-1,10-phenanthroline). Among these, insertion of the Ru-complex-modified lysine building block **9** turned out to be the method of choice (*Scheme 5*).

Introduction. – Fluorescence labelling of peptides is still an area of high interest. Chelate complexes of the lanthanides Eu^{III} and Tb^{III} are the most prominent label molecules for this purpose [1]. Their main advantage is represented by their strong fluorescence and excited-state lifetimes up to milliseconds which allow for time-resolved measurements virtually free of background.

For some time, our focus has been on ruthenium(II) charge-transfer complexes as alternative label entities. They combine high thermodynamic stability with chemical inertness and excited-state lifetimes in the microsecond range also allowing for highly sensitive time-resolved measurements [2].

Meanwhile, we have applied these complexes in combination with suitable donor and acceptor chromophores to robust fluorescence-resonance-energy-transfer (FRET) systems either in peptides or in synthetic DNA fragments [3]. The dye preferably applied to the labelling procedure is the [Ru^{II}(bathophenanthroline)] complex **1** depicted in *Fig. 1* (bathophenanthroline = 4,7-diphenyl-1,10-phenanthroline). The ligand of complex **1** carries sulfonate groups to mediate solubility in aqueous systems and one carboxy function which allows for attachments to amino functions of biomolecules *via* stable amide bonds. Recently, we were able to improve the synthesis of **1** substantially, and even a continuous large-scale preparation in a microreactor is now possible [4].

A severe drawback of our attachment procedures of Ru-complex **1** to peptides has been so far the restriction to N-terminal labelling[1][2]. To overcome this disadvantage and to extend the scope of application, allowing to place the Ru-complex at will into synthetic peptide sequences, we have contemplated three different solid-phase approaches, all of which were evaluated for labelling of the ε-amino group of L-lysine

[1] For incorporation of Eu^{III} complexes into peptides, see [5].

[2] For reviews on the preparation of peptide–metal complex conjugates, see [6].

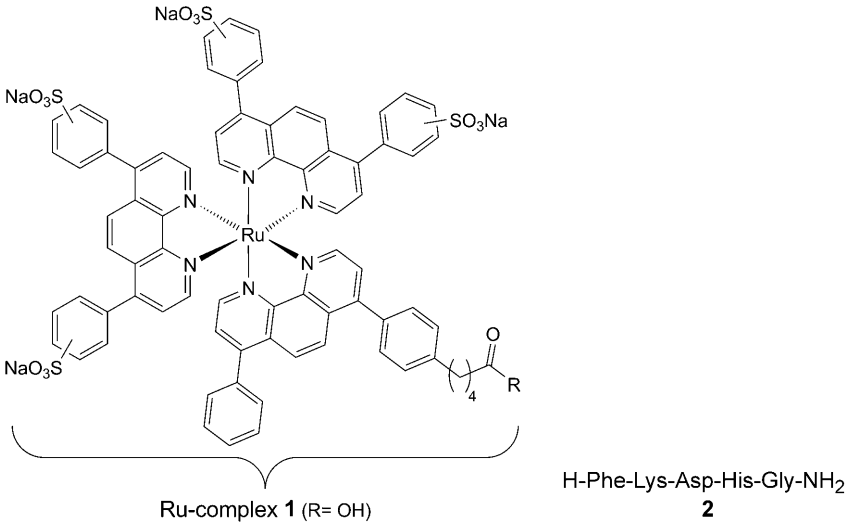
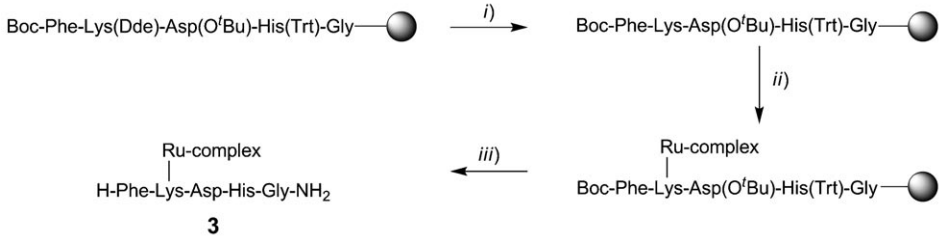


Fig. 1. *[Ru^{II}(bathophenanthroline)] complex* **1** *and model peptide* **2**

in the model peptide H–Phe–Lys–Asp–His–Gly–NH_2 (**2**) (*Fig. 1*). Compared to post-synthetic labelling procedures in solution, this would avoid final purification steps to remove excesses of unreacted label.

Results and Discussion. – In the first approach outlined in *Scheme 1*, the peptide was assembled employing the Fmoc/^{t}Bu strategy with the exception of L-lysine which was protected at the ε-amino function with the Dde protecting group developed by *Bycroft* and co-workers [7] (Fmoc = (3*H*-fluoren-9-ylmethoxy)carbonyl, Dde = 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl. Insertion of the last amino acid in the Boc-protected form and orthogonal removal of the Dde group of L-lysine with 2% hydrazine was followed by quantitative acylation of the side-chain amino group of Lys with Ru-complex **1** and TBTU as coupling reagent [8] (Boc = (*tert*-butoxy)carbonyl, TBTU = 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate).

Scheme 1. *Coupling of the Ru-Complex* **1** *to the ε-Amino Group of* L*-Lysine*



i) 2% Hydrazine in DMF, 4 × 3 min. *ii*) Ru-Complex **1**, TBTU, ^{i}Pr_2EtN, DMF 15 h. *iii*) $CF_3COOH/H_2O/$ ^{i}Pr_3SiH 95 : 2.5 : 2.5; 2 h.

Cleavage of the protecting groups and removal from the support with CF_3COOH yielded the desired labelled peptide **3**. Unfortunately, Ru-complex **1** revealed a high tendency to stick to the polymer matrix so that part of it was released only during this final CF_3COOH treatment and appeared as impurity in the desired labelled peptide **3** making an additional purification step necessary (*Fig. 2*). The quantitative acylation was confirmed by the absence of peptide **2** in the HPLC trace (t_R 19.8 min).

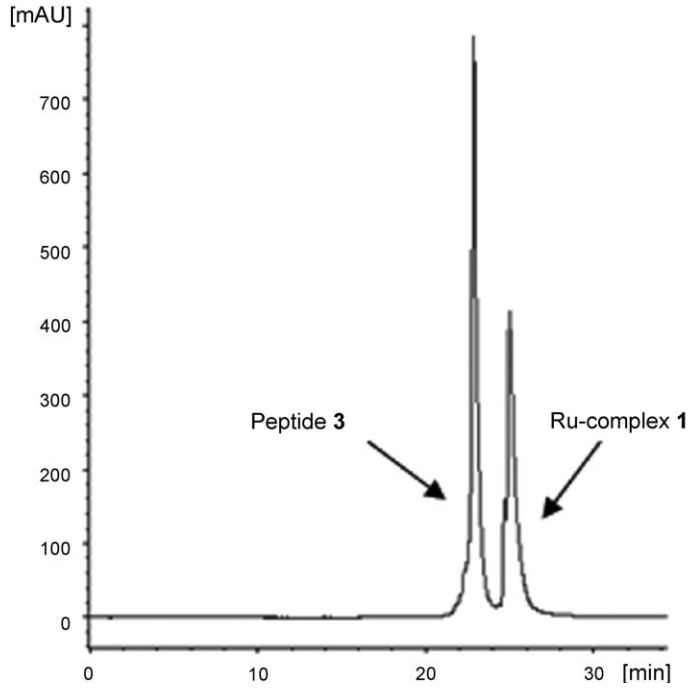
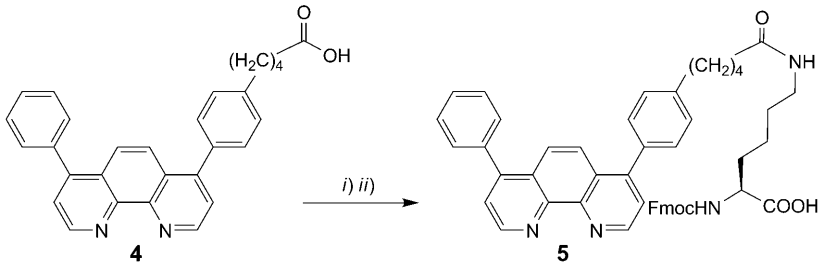


Fig. 2. *HPLC of crude peptide* **3**

The second approach was based on the introduction of a bathophenanthroline-modified L-lysine, **5**, during synthesis of the peptide. After assembly of the peptide chain, the bathophenanthroline entity should be turned on the solid support into the Ru-complex-labelled peptide **3**. The bathophenanthroline-modified L-lysine **5** was thus prepared from bathophenanthroline-derived ligand **4** and α-Fmoc-protected L-lysine according to *Scheme 2*. The carboxy function of ligand **4** had to be activated with HATU to reach a decent acylation rate [9] (HATU = 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate). The desired building block **5** was obtained in a yield of 48% after chromatography (silica gel) during which the bathophenanthroline entity severely hampered the purification procedure. The envisaged transfer of the solid-phase-attached bathophenanthroline-labelled peptide **6** into the aspired peptide **3** should then be performed by reaction with Ru-complex **7a** or **7b** according to *Scheme 3*. Initial experiments revealed that the exchange of the chloride ligands of **7a** by reaction with bathophenanthroline ligands proceeded very

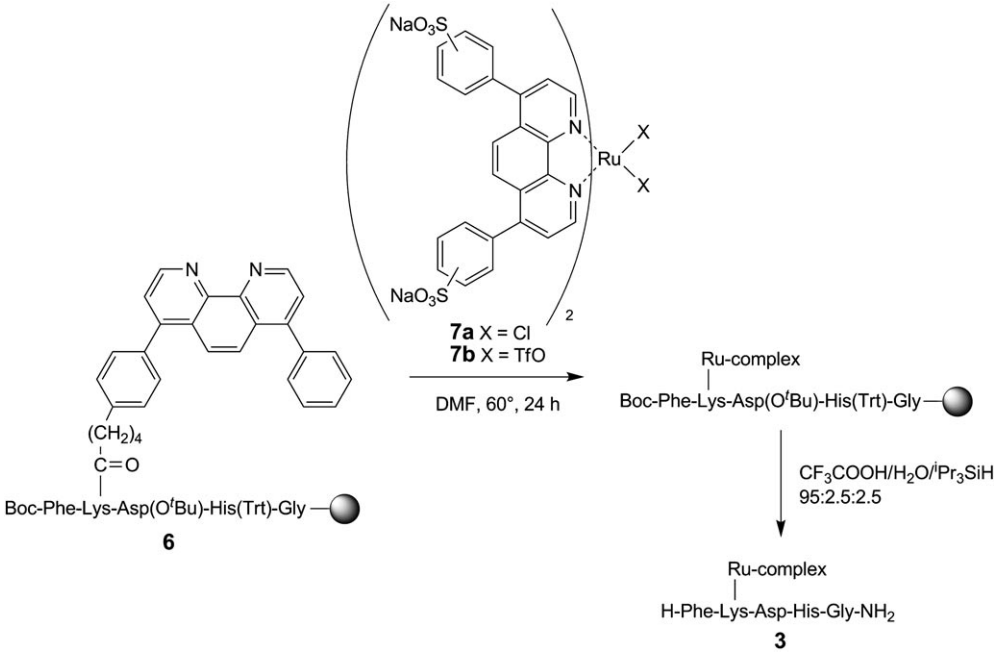
slowly and required high reaction temperatures not compatible with sensitive biomolecules. To overcome this limitation, we replaced in complex **7a** the chloride ions by the much weaker coordinating triflate ions (TfO^-)[3]) (→ **7b**). This was easily achieved by treating complex **7a** with silver triflate and filtering off the precipitated AgCl.

Scheme 2. *Synthesis of Ligand Building Block* **5**



i) HATU, $^{i}Pr_2EtN$, DMF, 15 min. *ii*) Fmoc-Lys-OH, 15 h.

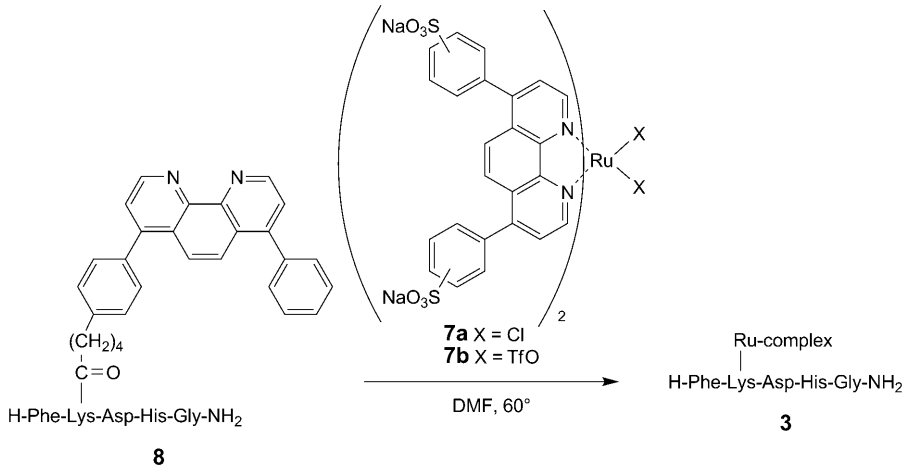
Scheme 3. *Complexation of Ligand Peptide* **6** *with Two Different Ru-Complexes,* **7a** *and* **7b**, *on Solid Support to Yield Peptide* **3**, *after Final Deprotection and Cleavage from the Solid Support*



[3]) We have so far no proof that the TfO^- ($CF_3SO_3^-$) is really coordinated or whether a solvent molecule (MeOH) takes its place instead.

A test reaction with peptide **8** in solution (*Scheme 4*) revealed indeed a much faster ligand-exchange reaction when **7b** instead of **7a** was applied for the transfer into the Ru-complex-labelled peptide **3**. The pertinent kinetic data are depicted in *Fig. 3*.

Scheme 4. *Complexation of Ligand Peptide* **8** *with Two Different Ru-Complexes* **7a** *and* **7b** *in Solution to Yield Peptide* **3**



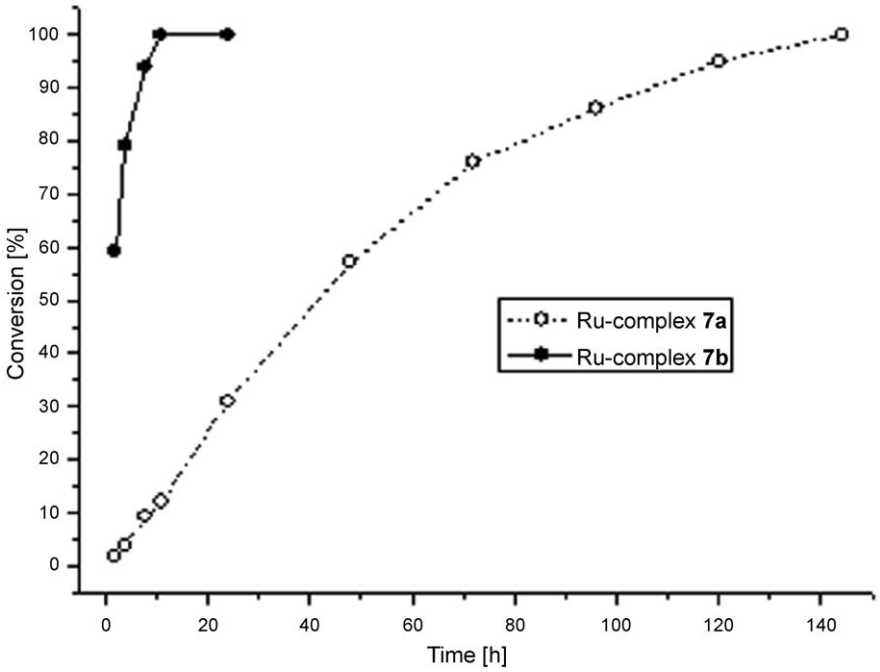


Fig. 3. *Kinetics for the formation of labelled peptide* **3** *after reaction of the bathophenanthroline-labelled peptide* **8** *with the Ru complexes* **7a** *or* **7b** *in solution*

After these positive results, the solid-phase-bound peptide **6** was treated with an excess of Ru-complex **7b** at 60° for 24 h. After removal of the excess of **7b** by intensive washing steps, the cleavage of the protecting groups and the removal from the support with CF_3COOH yielded crude peptide **3** (*Fig. 4*) without any trace of complex **7b** or peptide **8**. This indicated that the tendency to stick to the polymer matrix is much higher for Ru-complex **1** than that of the complex **7b**.

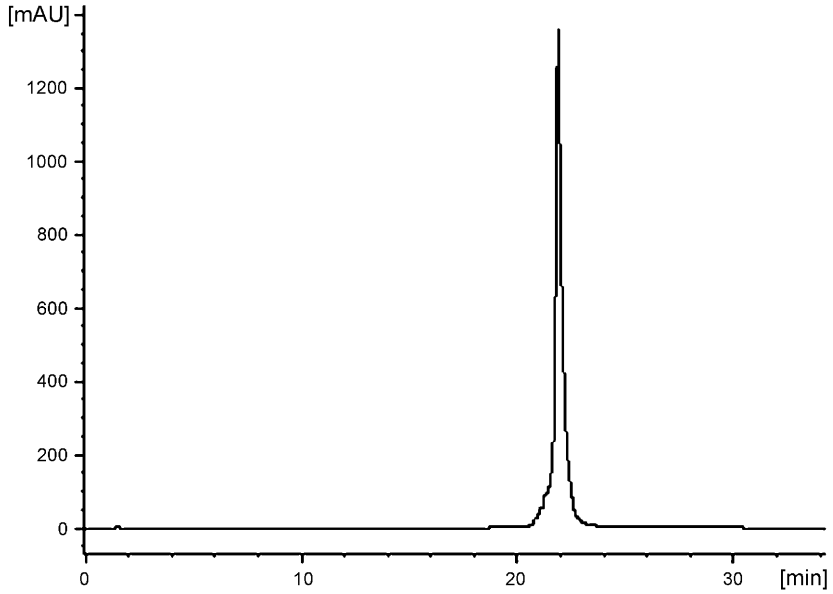


Fig. 4. *HPLC of crude peptide* **3** *after reaction of Ru-complex* **7b** *with the solid-phase-bound peptide* **6**

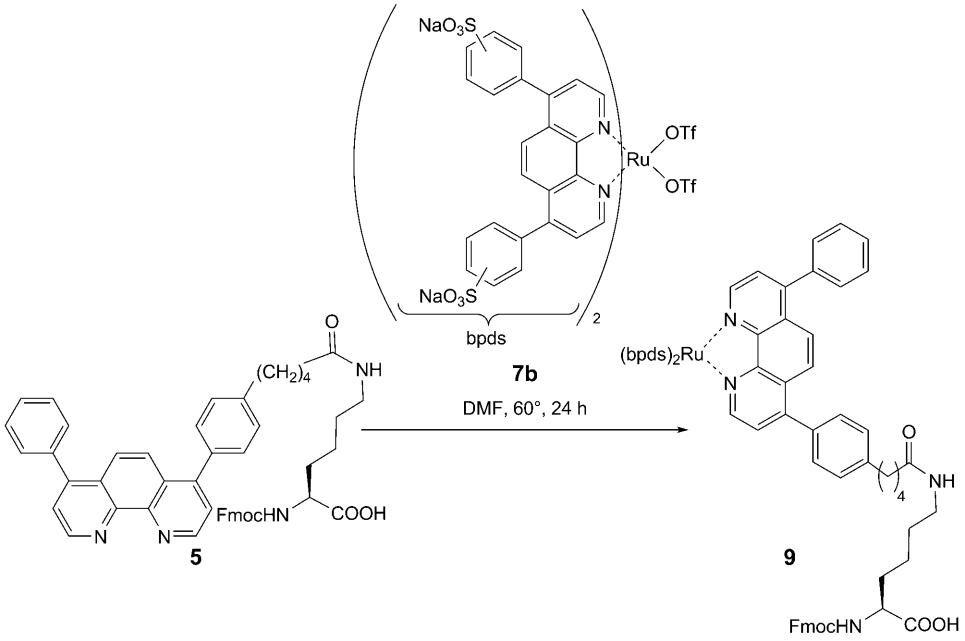
The ultimate strategy would be the application of a Ru-complex-labelled Fmoc-L-lysine building block during the assembly of the peptide on solid support. This would be compatible with automated peptide synthesis and require no additional modification procedures as in the previous two strategies. *Tor* and co-workers have recently reported on the preparation of a cysteine building block of a Ru-complex bearing two 1,1′-bis[pyridines] and phenanthroline as ligands [10]. Unfortunately, the reported strategy cannot be applied to the bathophenanthroline complexes. Therefore, we envisaged the synthesis of building block **9** for this purpose.

Initial attempts to prepare **9** *via* coupling of Ru-complex **1** to the free ε-amino group of Fmoc-Lys-OH failed due to difficulties in the separation of the Ru-complex **1** from the desired product **9**. Therefore, we prepared building block **9** in an alternative way starting from the bathophenanthroline-modified L-lysine derivative **5** and the Ru-complex **7b**[4]) according to *Scheme 5*. Purification of the crude material by chromatography (silica gel) was to no avail but prep. MPLC on reversed-phase silica gel (C_{18}) yielded the desired pure building block **9** in a yield of 79%. Next, peptide **3** was prepared employing standard Fmoc-building blocks in combination with Ru-complex-

[4]) **7a** as well as **7b** contained about 7% of $[Ru(bpds)_3]$ (see [4]).

modified building block **9** in the fourth position from the C-terminus. All couplings were performed with TBTU as activator. Final deprotection and removal from the support with CF_3COOH yielded the peptide without any by-products (*Fig. 5*) obviating additional purification steps and indicating at the same time impressively the high

Scheme 5. *Preparation of the Ru-Complex-Labelled* L-*Lysine Building Block* **9**



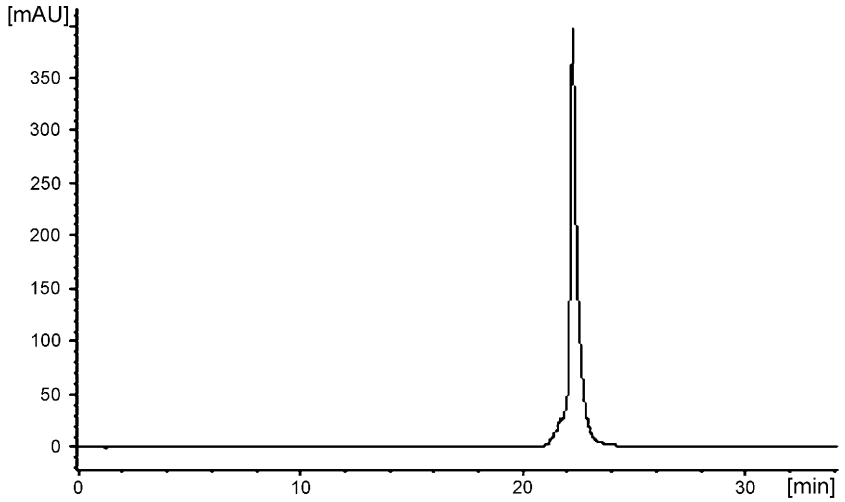


Fig. 5. *HPLC of crude peptide* **3** *obtained by the building-block approach with incorporation of* **9**

efficiency with which building block **9** had been incorporated. A slight excess of 1.2 equiv. of **9** had resulted in complete coupling.

Conclusions. – In this study, we evaluated three different strategies to increase the flexibility for the insertion of [Ru^{II}(bathophenanthroline)] complexes into peptides, which so far was limited to attachment at the N-terminus of peptides. All three methods are taking advantage of solid-phase chemistry allowing to remove excesses of reagents by washings. The first approach, based on an orthogonal protection of the ε-amino group of L-lysine with Dde yielded the desired labelled model peptide **3** together with Ru-complex **1** so that an additional purification was necessary. The second strategy, in which the complete Ru-complex was formed directly on the solid support from a bathophenanthroline-modified L-lysine of the peptide and complex **7b** yielded the desired peptide **3** with high efficiency and high purity. Finally, we were able to prepare a [Ru^{II}(bathophenanthroline)] complex – L-lysine building block **9** and were able to insert it into the peptide sequence during standard cycles and with high efficacy. Due to the high stability of the applied Ru-complexes towards CF_3COOH, no modifications or decompositions of the label were observed during deprotection. Since this last approach is compatible with automated peptide synthesis and requires no additional manipulations, it is recommended for the preparation of Ru-complex-labelled peptides.

Experimental Part

General. All reagents were purchased from commercial sources with the exception of compounds **1**, **4**, and **7a**, which were synthesized according to our established procedures [3]. The Ru-complex **7a** was converted to **7b** by dissolving **7a** in MeOH followed by addition of the appropriate amount of AgOTf. Amine-free DMF (*Roth*) was employed throughout Ru-complex and peptide synthesis. Peptide syntheses were carried out on a 0.02 mmol scale by the Fmoc/^{t}Bu protocol [11] and *Tentagel-S-RAM* resin (loading 0.24 mmol g^{-1}) with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) as a coupling reagent [8]. L-Aspartic acid as ^{t}Bu ester (Fmoc-Asp(O^{t}Bu)-OH) and L-histidine as trityl (Fmoc-His(Trt)-OH) side-chain-protected amino acids were employed. CC = column chromatography. HPLC: *Agilent-1100* system with a *Source-5RPC-ST-4.6/150* column (*Amersham Pharmacia Biotech*). MPLC: purification of the Ru-complex – amino acid **9** with a *Büchi* MPLC system (fraction collector *C660*, pump module *C605*, pump manager *C615*, and UV photometer *C635*); the C_{18} reversed-phase material for MPLC was synthesized by a modified standard procedure according to [12]. NMR Spectra: at 400 MHz (^{1}H), and at 100.6 MHz (^{13}C); chemical shifts δ in ppm rel. to the respective solvent signals, *J* in Hz. MS: *Finnigan MAT-8200* (EI), *TSQ-7000* (ESI); in *m*/*z*. HR-MS: *Bruker microToF-Q* equipped with a *Bruker Appollo* source; by the Institut Fédératif de Recherche 85 at the Louis Pasteur University, Strasbourg.

*(2*S*)-2-{[(9*H*-Fluoren-9-ylmethoxy)carbonyl]amino}-6-{{1-oxo-5-[4-(7-phenyl-1,10-phenanthrolin-4-yl)phenyl]pentyl}amino}hexanoic Acid* = N^2-*[(9*H*-Fluoren-9-ylmethoxy)carbonyl]*-N^6-*{1-oxo-5-[4-(7-phenyl-1,10-phenanthrolin-4-yl)phenyl]pentyl}*-L-*lysine*; **5**). Under Ar, a mixture of 5-[4-(7-phenyl-1,10-phenanthrolin-4-yl)phenyl]pentanoic acid (**4**; 1.00 g, 2.31 mmol, 1.0 equiv.), (2-1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU; 0.88 g, 2.31 mmol, 1.0 equiv.), and ^{i}Pr_2EtN (0.40 ml, 4.62 mmol, 2.0 equiv.) in DMF (20 ml) was stirred for 15 min at r.t. and then treated with Fmoc-Lys-OH (0.85 g, 2.31 mmol, 1.0 equiv.). The resulting suspension was diluted with DMF (40 ml) and stirred again for 15 h at r.t. (→ clear soln.). After evaporation of the solvent, the residue was suspended in H_2O (100 ml) and the mixture acidified with 2M HCl to a pH of 2. The resulting precipitate was filtered off, washed with H_2O (100 ml), and then purified by CC (SiO_2, CH_2Cl_2/MeOH/AcOH 98 : 2 : 0.1 → 90 : 10 : 0.1). From the pure fractions, AcOH was removed by co-evaporation with hexane: **5**

(0.88 g, 48%). Pale pink powder. ^{1}H-NMR (400 MHz, CD_3OD): 1.39–1.49 (*m*, $NHCH_2CH_2CH_2$); 1.50–1.59 (*m*, $CH_2CHCOOH$); 1.64–1.76 (*m*, $C_6H_4-CH_2CH_2CH_2$, $NHCH_2CH_2$); 2.21–2.27 (*m*, $NHCOCH_2$); 2.69–2.75 (*m*, $C_6H_4-CH_2$); 3.20 (*t*, *J* = 6.6, $NHCH_2$); 4.04 (*t*, *J* = 7.5, H–C(9) of Fmoc); 4.11–4.17 (*m*, CH–COOH); 4.18–4.23 (*m*, CH_2 of Fmoc); 7.20 (*dd*, *J* = 7.4, 4.6, H–C(2,7) of Fmoc); 7.27 (*d*, *J* = 7.4, H–C(4) of Fmoc); 7.29 (*d*, *J* = 7.4, H–C(5) of Fmoc); 7.37 (*d*, *J* = 8.1, H–C(3,5) of $C_6H_4(CH_2)_4$); 7.44 (*d*, *J* = 8.2, H–C(2,6) of $C_6H_4(CH_2)_4$); 7.51–7.58 (*m*, 7 arom. H); 7.64 (*d*, *J* = 7.3, H–C(3,6) of Fmoc); 7.77 (*d*, *J* = 4.9, H–C(3) of phen); 7.80 (*d*, *J* = 4.8, H–C(8) of phen); 7.88 (*d*, *J* = 9.5, H–C(1) of Fmoc); 7.92 (*d*, *J* = 9.5, H–C(8) of Fmoc); 9.08 (*d*, *J* = 4.9, H–C(2) of phen); 9.13 (*d*, *J* = 4.8, H–C(9) of phen). ^{13}C-NMR (100.6 MHz, CD_3OD): 24.32; 26.60; 29.92; 31.88; 32.34; 36.26; 36.87; 39.99; 55.30; 67.87; 68.11; 120.82; 125.74; 125.80; 125.95; 126.17; 126.20; 128.09; 128.71; 130.06; 130.14; 130.45; 130.79; 130.89; 135.26; 137.93; 142.37; 143.60; 143.74; 145.02; 145.18; 145.39; 148.75; 149.39; 152.56; 153.21; 158.59; 175.98. EI-MS: 805 (10, $[M+Na]^+$), 783 (100, $[M+H]^+$). Anal. calc. for $C_{50}H_{46}N_4O_5$: C 76.70, H 5.92, N 7.16; found: C 76.56, H 5.83, N 7.22.

Sodium Hydrogen {(2S)-2-{[(9H-Fluoren-9-ylmethoxy)carbonyl]amino}-6-{{1-oxo-5-[4-(7-phenyl-1,10-phenanthrolin-4-yl-$\kappa N^1,\kappa N^{10}$*)phenyl]pentyl}amino}hexanoato}bis{(1,10-phenanthroline-4,7-diyl-*$\kappa N^1,\kappa N^{10}$*)bis[benzenesulfonato](2–)}ruthenate(3–) Trifluoromethanesulfonate (4:1:1:2) (= Sodium Hydrogen {N^2-[(9H-Fluoren-9-ylmethoxy)carbonyl]-N^6-{1-oxo-5-[4-(7-phenyl-1,10-phenanthrolin-4-yl-*$\kappa N^1,\kappa N^{10}$*)phenyl]pentyl}-*L*-lysinato}bis{(1,10-phenanthroline-4,7-diyl-*$\kappa N^1,\kappa N^{10}$*)bis[benzenesulfonato](2–)}ruthenate(3–) 1,1,1-Trifluoromethanesulfonate (4:1:1:2)*; **9**·2 TfO^-). A soln. of the Ru-complex **7b** (465.7 mg, 0.304 mmol, 2.1 equiv.) and ligand **5** (250.0 mg, 0.319, 2.1 equiv.) in DMF (15 ml) was stirred at 60° for 24 h. The mixture was concentrated and purified by reversed-phase MPLC (C_{18}, H_2O/MeCN/CF_3COOH 85:15:0.1 → 67:33:0.1). Lyophylization of the combined product fractions gave **9**·2 TfO^- (0.522 g, 79%). Red powder. HR-ESI-MS: 932.1557 ($C_{100}H_{78}F_6N_8O_{23}RuS_6^{2-}$, $[M-4H-2OTf^-]^{2-}$; calc. 932.1567).

Sodium Bis{(1,10-phenanthroline-4,7-diyl-$\kappa N^1,\kappa N^{10}$*)bis[benzenesulfonato](2–)}{L-phenylalanyl-N^6-{1-oxo-5-[4-(7-phenyl-1,10-phenanthrolin-4-yl-*$\kappa N^1,\kappa N^{10}$*)phenyl]pentyl}-L-lysyl-L-α-aspartyl-L-histidylglycinamide}ruthenate(2–) Chloride (4:1:2)* (**3**·2 Cl^-) *by the Orthogonal Protecting Group Strategy.* Standard peptide synthesis was employed on a 0.02 mmol scale [11]. During peptide synthesis, L-lysine was introduced as the protected Fmoc-Lys(Dde)-OH and the last amino acid, L-phenylalanine, was introduced as Boc-Phe-OH. The Dde deprotection was accomplished *via* the addition of 2% (*v*/*v*) hydrazine/DMF (3 × 3 ml) and shaking for 3 min. For the coupling of the Ru-complex **1**·2 Cl^-, a soln. of **1**·2 Cl^- (52.5 mg, 0.03 mmol, 1.5 equiv.) in DMF (2 ml), TBTU (9.6 mg, 0.03 mmol, 1.5 equiv.), and ^{i}Pr$_2$EtN (27.4 µl, 0.16 mmol, 8.0 equiv.) were added to the resin, which was agitated for 15 h. Afterwards, the solid support was filtered and washed alternately with DMF/^{i}PrOH (5–3 ml), before the peptide was deprotected and cleaved from the solid support by exposure to $CF_3COOH/H_2O/^i Pr_3SiH$ 95:2.5:2.5 for 2 h. The cleavage cocktail was treated with Et_2O to precipitate peptide derivative **3**·2 Cl^- and Ru-complex **1**·2 Cl^-. Isolation of **3**·2 Cl^- was finally achieved by prep. HPLC. ESI-MS: 1051 (100, $[M-2Cl-4Na+4H]^{2+}$).

L-Phenylalanyl-N^6-{1-oxo-5-[4-(7-phenyl-1,10-phenanthrolin-4-yl)phenyl]pentyl}-L-lysyl-L-α-aspartyl-L-histidylglycinamide (**8**). Standard peptide synthesis was employed on a 0.02 mmol scale. As fourth amino acid, the ligand amino acid **5** (20.4 mg, 0.026 mmol, 1.3 equiv.) was introduced with TBTU (10.9 mg, 0.026 mmol, 1.3 equiv.) and ^{i}Pr$_2$EtN (27.4 µl, 0.16 mmol, 8.0 equiv.) in DMF (2 ml). For this amino acid, the coupling time was extended to 15 h. The ligand-containing peptide **8** was isolated as a pale pink powder. ESI-MS: 1017 (100, $[M+H]^+$).

Sodium Ruthenate(2–) Chloride (4:1:2) **3**·*2 Cl$^-$ or Sodium Ruthenate(2–) Trifluoromethanesulfonate (4:1:2)* **3**·*2 TfO$^-$ by Complexation of Peptide* **8** *with the Ru-Complex* **7a** *or* **7b** *in Solution.* Two *Eppendorf* tubes were filled in parallel with stock solns. of Ru-complex **7a** or **7b** (*c* = 0.015M, 0.18 ml, 2.7 µmol, 1.5 equiv.), ligand peptide **8** (2.0 mg, 1.8 µmol, 1.0 equiv.), and DMF (0.8 ml). The two *Eppendorf* tubes were agitated in a thermomixer at 60° and 600 rpm. The samples were analyzed by HPLC to determine the complexation efficiency. Isolation of **3**·2 X^- ($X^- = Cl^-$ or TfO^-) was finally achieved by prep. HPLC. ESI-MS: 1051 (100, $[M-2X-4Na+4H]^{2+}$).

Sodium Bis{(1,10-phenanthroline-4,7-diyl-$\kappa N^1,\kappa N^{10}$*)bis[benzenesulfonato](2–)}{L-phenylalanyl-N^6-{1-oxo-5-[4-(7-phenyl-1,10-phenanthrolin-4-yl-*$\kappa N^1,\kappa N^{10}$*)phenyl]pentyl}-L-lysyl-L-α-aspartyl-L-histidyl-*

glycinamide}ruthenate(2–) 1,1,1-Trifluoromethanesulfonate (4:1:2) (**3**·2 TfO^-) *by Complexation of Peptide* **6** *with the Ru-Complex* **7b** *on Solid Support.* Peptide **6** was synthesized on a 0.02 mmol scale on solid support. After coupling of Boc-Phe-OH as last amino acid, a stock soln. of Ru-complex **7b** (c = 0.015M, 2.0 ml, 30 μmol, 1.5 equiv.) was added. The resin was agitated in a thermomixer at 60° and 600 rpm for 24 h. The solid support was filtered and washed alternately with DMF/^{i}PrOH (5 × 3 ml), before the peptide was deprotected and cleaved from the solid support by exposure to $CF_3COOH/H_2O/{}^iPr_3SiH$ 95:2.5:2.5 for 2 h. Addition of Et_2O precipitated **3**·2 TfO^- which was obtained in pure form after lyophilization (8.2 mg, 19%). Red powder. ESI-MS: 1051 (100, $[M - 2\,TfO - 4\,Na + 4\,H]^{2+}$).

Sodium Ruthenate(2–) Trifluoromethanesulfonate (4:1:2) **3**·*2 TfO^- from Amino Acid Derived Ru-Complex* **9**·*2 TfO^-*. Standard peptide synthesis was employed on a 0.02 mmol scale but as fourth amino acid, the amino acid derived Ru-complex **9**·2 TfO^- (45.0 mg, 0.024 mmol, 1.2 equiv.) in DMF (2 ml), TBTU (7.7 mg, 0.024 mmol, 1.2 equiv.), and $^iPr_2EtN$ (27.4 μl, 0.16 mmol, 8.0 equiv.) were added to the resin, which was agitated for 15 h. The solid support was filtered and washed alternately with DMF/^{i}PrOH (5 × 3 ml). Deprotection and cleavage from the solid support was performed by exposure to $CF_3COOH/H_2O/{}^iPr_3SiH$ 95:2.5:2.5 for 2 h. After precipitation with Et_2O and lyophilization, **3**·2 TfO^- was obtained (15.4 mg, 35%). Red powder. ESI-MS: 1051 (100, $[M - 2\,TfO - 4\,Na + H]^{2+}$).

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