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Synthesis and characterisation of a ruthenocenoyl bioconjugate with the cyclic octapeptide octreotate

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Dedicated, in Eb[7], to Professor Dr. Christoph Elschenbroich on the occasion of his 70th birthday

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ABSTRACT

The reaction of activated ruthenocene carboxylic acid with the resin-bound peptide octreotate yields, after cleavage and purification by preparative HPLC, the first ruthenocenoyl peptide bioconjugate **1**. Octreotate is a chemically stabilized analogue of somatostatine. It is a cyclic octapeptide with a disulfide bond and has been previously used for molecular diagnostics due to the fact that somatostatine receptors are over-expressed by a variety of cancer cells. Conjugate **1** was obtained in good yield and purified by preparative HPLC to >95% purity as judged by analytical HPLC. It has been identified by HPLC, IR and mass spectrometry (ESI and MALDI-TOF). The peptide's NMR signals are assigned by standard 2D methods. In addition, the ^{1}H NMR spectrum of **1** shows characteristic signals for the metallocene between 5.1 and 4.3 ppm. Compound **1** thus is a new example of tumor-targeted organometallic ruthenium bioconjugates.

1. Introduction

Conjugates of biomolecules with organometallic complexes are receiving increased attention for applications in biosensors, molecular diagnostics, and analytical tools. By far the most frequently used metal complex for biosensor applications is ferrocene (dicyclopentadienyl iron, Cp_2Fe) [1–5]. This is due to the ease of synthesis of functionalized derivatives as well as its favourable electronic properties, in particular the high stability of the +II and +III oxidation states. The electronic structure of the paramagnetic ($S = ½$) +III oxidation state has been studied in detail by EPR spectroscopy [6–8]. Its properties have been compared by Elschenbroich and coworkers to their open-ring analogues and an explanation of the differences has been offered based on electronic structure calculations [9].

Given the abundance of ferrocene bioconjugates, we note with astonishment the almost complete lack of biological applications of other metallocenes with the ferrocene structure. A few cobaltocenium conjugates with peptide nucleic acid oligomers (PNA) [10,11] and peptides [12–15] have been published by our group. Ruthenocene has been mentioned in a few early papers exclusively in the context of radiolabelling with ^{103}Ru [16–19], and more recently labelling of estradiol by ruthenocene was proposed by Jaouen and coworkers [20]. Gmeiner et al. have recently reported ruthenocene derivatives of dopamine receptor ligands which show an increased affinity and specificity for the D4 dopamine receptor subtypes compared to their metal-free congeners [21]. Organometallic Ru(II) arene compounds alone, on the other hand, were investigated by several groups for their anti-proliferative properties, making them promising anti-cancer drug candidates [22–26].

In this work, we report the first ruthenocene peptide bioconjugate, in which ruthenocene carboxylic acid is coupled to the N-terminus of the cyclic octapeptide ocreotate by a peptide bond. Octreotate is an analogue of the naturally occurring peptide somatostatine. Compared to somatostatine, octreotate has a shortened amino acid sequence, shows enhanced stability under biological conditions, and a higher affinity to the somatostatine receptor subtypes 2 [27,28]. Somatostatine receptors (SSTRs) are over-expressed on several tumors and have been successfully targeted for imaging, especially with ^{99m}Tc and ^{111}In (Octreoscan®) [29,30]. The challenge of this work was to establish suitable conditions of solid phase peptide synthesis (SPPS) that will allow the incorporation of a ruthenocene derivative as part of the SPPS cycle, which in the case of octreotate includes a further step for formation of the disulfide bond.

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2. Experimental section

2.1. Materials

Ruthenocene carboxylic acid was synthesized according to literature procedures [31–33]. Chemicals and solvents were used as received from commercial suppliers. Only enantiomerically pure amino acids were used throughout, absolute chirality is given.

2.2. Instrumentation and analytical measurements

A Liberty Microwave Peptide Synthesizer from CEM was used for peptide synthesis. HPLC analysis and purifications were carried out using C18 analytical (Varian Dynamax, 4.5 mm × 250 mm) and C18 semipreparative (Varian Dynamax, 21.4 mm × 250 mm) columns on a customized Varian Prostar Instrument. IR data were collected on a Bruker Tensor 27 with an ATR unit. ESI-MS analyses were performed on a Bruker Esquire 6000 instrument. The matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) mass spectra were measured on a Bruker Daltonics Autoflex. The experiments were performed in linear mode with positive polarity using sinapinic acid as the matrix. Nuclear magnetic resonance spectra were recorded on a Bruker DRX 600 MHz spectrometer. ^{1}H and ^{13}C chemical shifts are given in ppm and were referenced with the residual solvent resonances relative to tetramethylsilane (TMS). Lyophilization was performed on a Alpha 1-4 LD plus lyophilizator from Christ.

2.3. Synthesis of ruthenocenoyl-octreotate conjugate 1

Fmoc-Octreotate was synthesized on an automated peptide synthesizer by solid phase methods using a 0.25 mmol scale Fmoc-strategy on Fmoc-Thr(^{t}Bu)-Wang resin (0.63 mmol/g, Iris Biotech) generating the C-terminal carboxylic acid after cleavage. The resin-bound peptide sequence was D-Phe-Cys(Acm)-Phe-D-Trp-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(tBu)-Wang-resin. Stepwise coupling reactions were performed with enantiomerically pure Fmoc-protected amino acids (IRIS Biotech GmbH or Novabiochem), 1-hydroxybenzotriazole (HOBt), 2-(1H-benzotriazol-1-yl)-1,3,3-tetramethyl-uroniumtetrafluoroborate (TBTU), diisopropyl-ethylamine (DIPEA), (4:4:4:6 equiv., cystein coupling: 50 °C, 0 W, 120 s, followed by 50 °C, 25 W for 240 s. Other amino acids were coupled using: 75 °C, 24 W, 300 s). 2-fold N-terminal deprotection of the Fmoc group was performed using 20% piperidine solution in DMF (first cycle: 75 °C, 35 W, 30 s; second cycle 75 °C, 50 W, 180 s). A total of 0.055 mmol of the resin-bound side-chain protected Fmoc-Octreotate was transferred into a batch reactor, followed by cyclization at room temperature with a 2-fold molar excess of thallium(III)trifluoroacetate ($Tl(TFA)_3$) in DMF for 1 h [34]. After washing with DMF, ruthenocene carboxylic acid (0.22 mmol, 0.0605 g) was coupled using O-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorophosphate (HATU) (0.22 mmol, 0.084 g), 56.4 μL DIPEA (4:4:6) in 500 μL NMP for 3 h. The completeness of ruthenocene carboxylic acid coupling was determined by Kaiser's test [35]. The resin was washed with DMF and DCM, shrinked with MeOH and dried under vacuum for 30 min. Finally, cleavage of the bioconjugate from the resin was performed with TFA/phenol/triisopropylsilane (TIS) (2 mL, 85:10:5) for 2 h at room temperature. The resin was filtered and washed with 0.5 mL TFA. Addition of cold diethyl ether yielded a beige precipitate, which was washed repeatedly with diethyl ether. The product was dissolved in acetonitrile/water, filtered and lyophilized. The crude yield was 75% (0.053 g), see Fig. 1. The bioconjugate was purified by RP-HPLC using a gradient of acetonitrile/water containing 0.1% TFA (60 min, 4 mL/min). The fractions containing the conjugate were collected and lyophilized. The purity of the conjugate was 95% as determined by analytical HPLC (Fig. 2). IR (neat, ATR): 3293 (br, ν_{NH}), 1643 (s, $\nu_{CO(1)}$), 1526 (m, $\nu_{CO(2)}$). MALDI-TOF MS: *m/z* 1289.9 $(M+H)^+$; ESI-MS (pos.): 1291.22 $(M+3H)^+$, Calc. for $C_{60}H_{70}N_{10}O_{12}RuS_2$: 1288.4; ^{1}H NMR (DMSO-d_6, 600 MHz): δ 10.77 ($1H_{\varepsilon 1,\ Trp4}$), 8.88 ($1H_{NH,\ Cys2}$), 8.74 ($1H_{NH,\ Trp4}$), 8.52 ($1H_{NH,\ Phe3}$), 8.46 ($1H_{NH,\ Cys7}$), 8.42 ($1H_{NH,\ Lys5}$), 8.31 ($1H_{NH,\ Thr8}$), 7.64 ($3H_{\zeta,\ Lys5}$), 7.57 ($1H_{NH,\ Thr6}$), 7.48 ($1H_{NH,\ Phe1}$), 7.46 ($1H_{\varepsilon 3,\ Trp4}$), 7.35 ($2H_{\delta,\ Phe1}$), 7.34 ($1H_{\zeta 2,\ Trp4}$), 7.28 ($2H_{\varepsilon,\ Phe1}$), 7.18 ($1H_{\zeta,\ Phe1}$), 7.12 (3H, $2H_{\varepsilon,\ Phe3}$, $1H_{\zeta,\ Phe3}$), 7.07 ($1H_{\eta 2,\ Trp4}$), 7.04 ($2H_{\delta,\ Phe3}$), 6.99 ($1H_{\zeta 3,\ Trp4}$), 6.97 ($1H_{\delta 1,\ Trp4}$), 5.32 ($1H_{\alpha,\ Cys7}$), 5.27 ($1H_{\alpha,\ Cys2}$), 5.14 ($1H_{Cp2}$), 5.06 ($1H_{Cp5}$), 4.94 ($1H_{\alpha,\ Phe1}$), 4.82 ($1H_{\gamma 1,\ Thr6}$), 4.63 (3H, $2H_{Cp3,4}$, $1H_{\alpha,\ Phe3}$), 4.55 ($1H_{\alpha,\ Thr6}$), 4.34 (6H, $1H_{\beta,\ Thr8}$, $2H_{Cp'}$), 4.26 (2H, $1H_{\alpha,\ Thr8}$, $1H_{\alpha,\ Trp4}$), 4.12 ($1H_{\gamma 1,\ Thr8}$), 4.03 ($1H_{\alpha,\ Lys5}$), 3.97 ($1H_{\beta,\ Thr6}$), 3.15 ($1H_{\beta 2,\ Phe1}$), 2.98 ($1H_{\beta 2,\ Trp4}$), 2.92 ($1H_{\beta 3,\ Phe1}$), 2.87 (3H, $H_{\beta 2,\ Cys2}$, $H_{\beta 2,\ Cys7}$, $H_{\beta 2,\ Phe3}$), 2.85 ($1H_{\beta 3,\ Phe3}$), 2,82 (2H, $H_{\beta 3,\ Cys2}$, $H_{\beta 3,\ Cys7}$), 2.75 ($1H_{\beta 3,\ Trp4}$), 2.61 ($2H_{\varepsilon,\ Lys5}$), 1.75 ($1H_{\beta 2,\ Lys5}$), 1.32 (3H, $1H_{\beta 3,\ Lys5}$, $2H_{\delta,\ Lys5}$), 1.20 ($3H_{\gamma 2,\ Thr8}$), 1.06 ($3H_{\gamma 2,\ Thr6}$), 0.83 ($2H_{\gamma,\ Lys5}$). ^{13}C NMR (DMSO-d_6, 150 MHz): δ 172.7 (CO, $_{Phe1}$), 171.8 (CO, $_{Trp4}$), 171.6 (CO, $_{Thr8}$), 170.8 (CO, $_{Lys5}$), 170.3 (CO, $_{Phe3}$), 170.0 (CO, $_{Thr6}$), 169.6 (CO, $_{Cys7}$), 168.6 (CO, $_{Cys2}$), 167.1 (CO, Cp), 138.0 ($C_{\gamma,\ Phe1}$), 136.4 ($C_{\gamma,\ Phe3}$), 135.8 ($C_{\varepsilon 2,\ Trp4}$), 129.0 ($C_{\delta,\ Phe1}$), 128.6 ($C_{\delta,\ Phe3}$), 127.7 ($C_{\varepsilon,\ Phe1}$), 127.6 ($C_{\varepsilon,\ Phe3}$), 126.9 ($C_{\delta 2,\ Trp4}$), 126.0 ($C_{\zeta,\ Phe1}$), 125.9 ($C_{\zeta,\ Phe3}$), 123.3 ($C_{\delta 1,\ Trp4}$), 120.6 ($C_{\eta 2,\ Trp4}$), 117.9 ($C_{\zeta 3,\ Trp4}$), 117.8 ($C_{\varepsilon 3,\ Trp4}$), 111.0 ($C_{\zeta 2,\ Trp4}$),

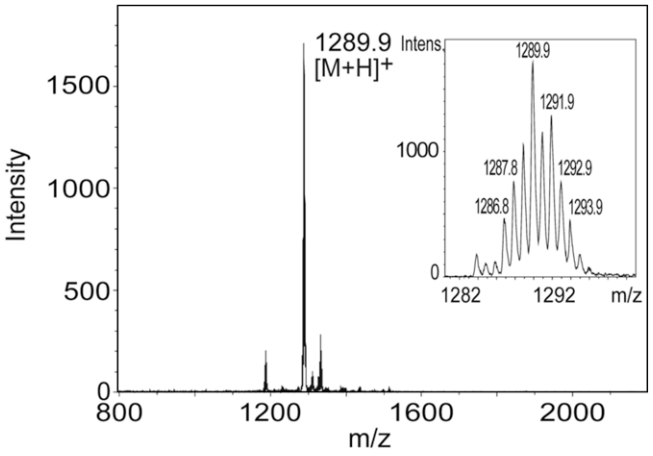


Fig. 1. MALDI-TOF of the crude ruthenocenoyl-octreotate **1**. The inset shows the experimentally observed isotope pattern of the $[M+H]^+$ peak.

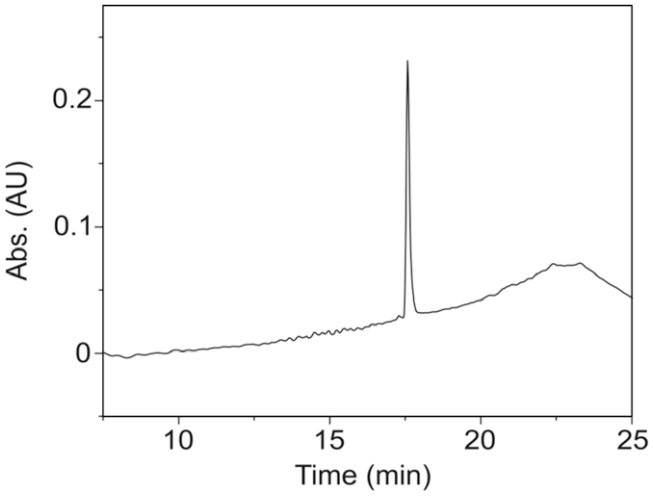


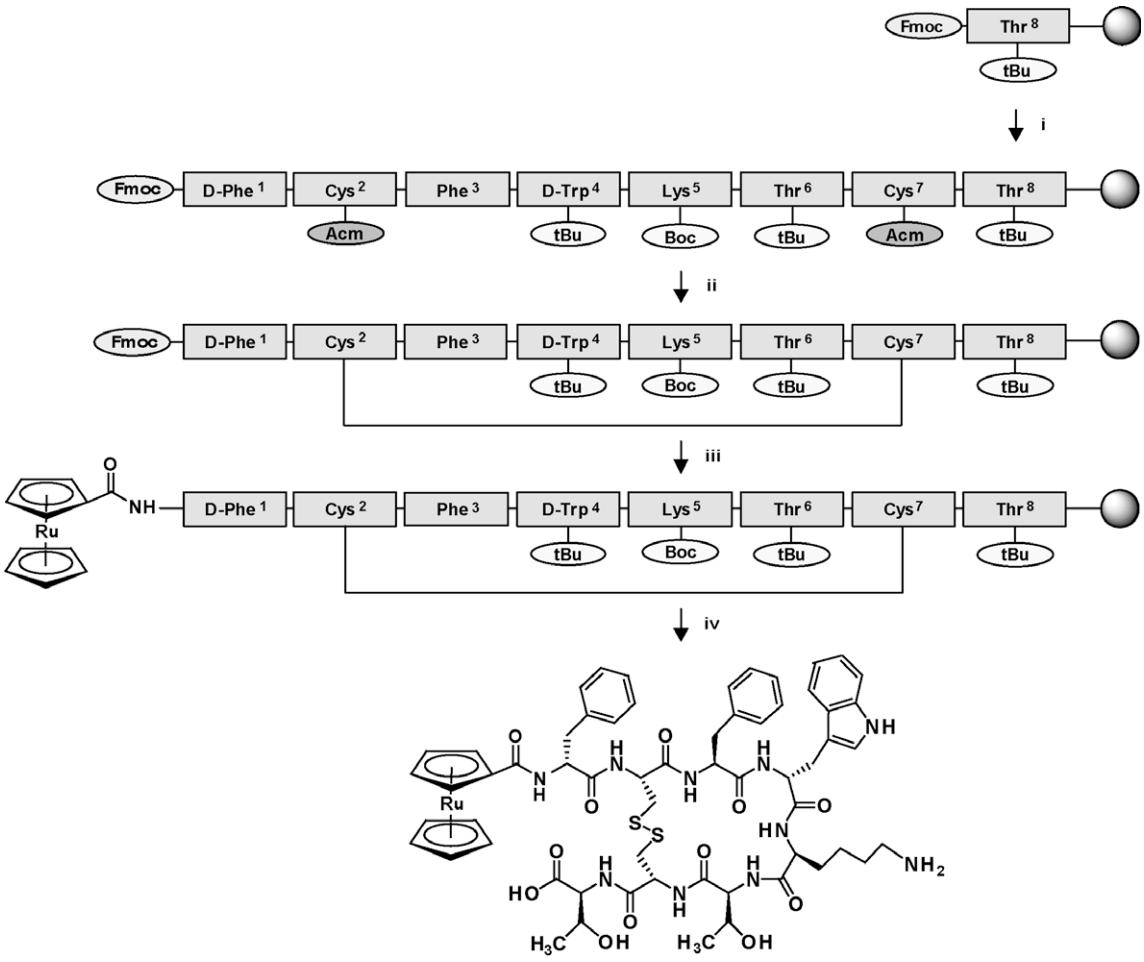
Fig. 2. HPLC trace (220 nm) of the purified ruthenocenoyl-octreotate **1**.

108.8 ($C_{\gamma,\ Trp4}$), 71.5 ($C_{Cp3,4}$), 71.0 ($C_{Cp'}$), 70.2 (C_{Cp5}), 69.2 (C_{Cp2}), 67.4 ($C_{\beta,\ Thr8}$), 67.0 ($C_{\beta,\ Thr6}$), 58.1 ($C_{\alpha,\ Thr8}$), 57.7 ($C_{\alpha,\ Thr6}$), 54.9 ($C_{\alpha,\ Trp4}$), 53.5 ($C_{\alpha,\ Phe3}$), 53.2 ($C_{\alpha,\ Phe1}$), 52.2 ($C_{\alpha,\ Lys5}$), 52.1 ($C_{\alpha,\ Cys2}$), 51.6 ($C_{\alpha,\ Cys7}$), 44.9 ($C_{\beta,\ Cys7}$), 44.1 ($C_{\beta,\ Cys2}$), 38.5 ($C_{\beta,\ Phe3}$), 38.3 ($C_{\varepsilon,\ Lys5}$), 38.0 ($C_{\beta,\ Phe1}$), 30.2 ($C_{\beta,\ Lys5}$), 26.2 ($C_{\delta,\ Lys5}$), 25.8 ($C_{\beta,\ Trp4}$), 21.7 ($C_{\gamma,\ Lys5}$), 20.1 ($C_{\gamma,\ Thr6}$), 20.0 ($C_{\gamma,\ Thr8}$).

3. Results and discussion

Ruthenocene carboxylic acid was prepared by following the literature procedure [31–33]. The octreotate peptide was prepared by solid–phase peptide synthesis on an automated, micro-wave assisted synthesizer. The complete peptide sequence was assembled in the synthesizer. The resin was then transferred into a batch reactor which consists of a syringe with a frit at the bottom. All subsequent steps were performed manually. The Acm-protecting groups were removed oxidatively and the thiol groups of both cysteine residues were oxidized with $Tl(TFA)_3$ [34] to form an intra-peptide disulfide bridge. Subsequently, the N-terminal Fmoc group was removed and ruthenocene carboxylic acid was coupled to form the the ruthenocenoyl-octreotate conjugate **1** (Scheme 1).

To this end, the free resin-bound octreotate was reacted with ruthenocene carboxylic acid in NMP in the presence of HATU and DIPEA as activators and coupling reagents. The conjugate was successfully cleaved from the resin by using 85% TFA, which left the metallocene unit untouched, as shown by a MALDI-TOF mass spectrum of the crude product (see Fig. 1). This figure also indicates that even the raw material is of good purity already. The conjugate was purified by reverse-phase HPLC on a C18 column and found to be 95% pure by subsequent analytical HPLC (see Fig. 2). After lyophilyzation, an off-white powder was obtained. The identity of the purified product was verified by mass spectrometry (ESI and MALDI-TOF), IR and NMR spectroscopy using H,H-TOCSY, C,H-COSY and HMBC-spectra. The experimental MALDI-TOF MS data is in agreement with the calculated values of $[M+H]^+$ and display the proper isotopic mass distribution pattern (see Fig. 1). However, the observed $[M+3H]^+$ mass in the ESI-MS rather seems to indicate reduction of the disulfide bond. Independent confirmation of an intact disulfide bond in **1**, and thus reduction under the conditions of electrospray ionisation, is obtained from NMR analysis. The chemical shift of the β-carbon atoms of cystein can be used to determine the oxidation status of disulfide bridged peptides [34]. Oxidation of cysteine to cystine leads to a downfield shift of the C_β atoms of cystein. The ^{13}C NMR signals of the two C_β of cystine in octreotate are observed around 46.5 ppm according to Mier et al. [34], indicating formation of the disulfide bond, whereas the nonoxidized C_β of cysteines shows an average shift of 34 ppm [36]. For compound **1**, all peptide signals were assigned with the help of standard 1 and 2D homo- and heteronuclear correlation experiments. Conjugate **1** shows a C_β ^{13}C NMR chemical shift of 44.1 and 44.9, thus indicating a disulfide bond formation. Furthermore, the ^1H NMR signals of the metallocene around 5 ppm provide a metal-specific probe. Integration of these signals further confirms the proposed



Scheme 1. Preparation of ruthenocenoyl-octreotate. Reagents and conditions: (i) stepwise peptide couplings on the synthesizer (see experimental); (ii) formation of the disulfide bond: $Tl(TFA)_3$ in DMF; (iii) ruthenocene carboxylic acid, HATU in NMP and (iv) cleavage: TFA, phenol, TIS.

composition of **1**. Also, the IR spectrum of **1** shows no SH signal between 2550 and 2600 cm^{-1}.

4. Conclusions

This paper reports the first ruthenocenoyl peptide bioconjugate, which has been synthesized by solid phase peptide synthesis methods. Octreotate, a chemically stabilized peptide analogue of somatostatine, has been chosen for the purpose of targeted drug delivery to somatostatin receptor-positive tumor cells. In this conjugate, the peptide moiety thus serves as tumor targeting unit. Although similar in structure, the chemical properties of ruthenocene are distinctly different from ferrocene, which has been frequently employed in previous work on all kinds of bioconjugates [1,3,4,37]. By substituting ferrocene for the ruthenocene moiety, we thus expect to shed further light on the biological properties and physiological activity of this interesting class of organometallic compounds. Work along these lines is in progress in our laboratory.

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