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Ruthenium-based bioconjugates: Synthesis and X-ray structure of the mixed ligand sandwich compound $RuCp^{iPr}(p\text{-}(CO_2H)C_6H_4Tp)$ and labelling of amino acids and the neuropeptide enkephalin

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Dedicated to Professor Gérard Jaouen on the occasion of his 65th birthday

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ABSTRACT

Four differently substituted mixed ligand sandwich complexes $CpRu(p\text{-}BrC_6H_4)Tp$ (**3**), $CpRu(p\text{-}BrC_6H_4)Tp^{Me}$ (**4**), $Cp^*Ru(p\text{-}BrC_6H_4)Tp$ (**5**), $Cp^{iPr}Ru(p\text{-}BrC_6H_4)Tp$ (**6**), incorporating cyclopentadienyl (Cp) and functionalized tris(pyrazolyl)borate (Tp) ligands, have been synthesized and characterized. Air-stable **6** has been converted to benzoic acid-functionalized $Cp^{iPr}Ru(p\text{-}(CO_2H)C_6H_4)Tp$ (**7**), which has been structurally characterized in the solid state by X-ray diffraction. Compound **7** may be readily coupled to biomolecules as exemplified by the coupling to phenylalanine-methylester to give $Cp^{iPr}Ru(p\text{-}(CO\text{-}Phe\text{-}OMe)C_6H_4Tp)$ (**8**). In a solid phase peptide synthesis (SPPS), **7** has been coupled to the pentapeptide Enkephalin, to provide $Cp^{iPr}Ru(p\text{-}(CO\text{-}Tyr\text{-}Gly\text{-}Gly\text{-}Phe\text{-}Leu\text{-}OH)C_6H_4Tp)$ (**9**) as the first example of a mixed ligand sandwich ruthenium bioconjugate.

1. Introduction

The labelling of biologically active molecules (e.g. DNA, RNA, peptides, drugs) by metal containing compounds can alter or enhance their properties decisively. While the labelling with coordination compounds has been employed in a manifold of applications ranging from drugs and radiochemical imaging agents to heavy atom probes for spectroscopy [1], the use of organometallic compounds for similar purposes is limited due to their lower stability under physiological conditions (aqueous media, oxygen atmosphere). Nevertheless, the field of bioorganometallic chemistry has emerged as an important research topic in the recent years offering new opportunities in medicinal and biochemical applications [2,3]. Consequently, there is a steady quest for compounds that are air- and waterstable and allow the facile attachment of biomolecules. One class of compounds that has been intensively investigated and employed in this area is that of the original metallocene Cp_2Fe and its heavier congener Cp_2Ru and their derivatives [4,5]. Notably, the analogues of these metallocenes, whether homoleptic or heteroleptic, have not been studied to any serious extent. As such, we have been interested in exploiting coordination complex analogues of group 8 metallocenes and their potential applications within bioorganometallic chemistry. A ligand system which has been frequently employed as a Cp surrogate is the tris(pyrazolyl)borate (Tp, also classified as "scorpionate") [6], because they are both isoelectronic [7]. Furthermore recent research in our group revealed 4-bromophenyl-tris(pyrazolyl)borate ($p\text{-}BrC_6H_4Tp$ or Tp′), a functionalised scorpionate ligand [6,8], to be a versatile precursor in the synthesis of transition metal bioconjugates [9,10]. Consequently, we were curious in employing Tp′ ligands in bioorganometallic metallocene analogues.

We considered ruthenium over iron for the ease of synthesis and characterization. For example, the synthesis of mixed ligand ferrocene analogues like CpFeTp is reported to be complicated [11], whereas a rather rich chemistry is found for analogue ruthenium compounds [11–13]. Further it has to be noticed, that the readily available $FeTp_2$ is known for temperature dependent spin-crossover behaviour [14], and that this behaviour can be triggered by small changes to the substituent on the boron, as reported for example for $Fe(p\text{-}IC_6H_4Tp)_2$ [15].

Moreover, regarding the recent developments in the field of ruthenium based bioorganometallics [16,17], in particular the possible anticancer properties of compounds such as RAPTA [18,19], we were interested in mixed ligand sandwich ruthenocene

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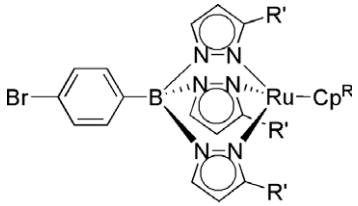


R'=H, Cp^R=Cp **3**
R'=CH_3, Cp^R=Cp **4**
R'=H, Cp^R=Cp* **5**
R'=H, Cp^R=Cp^{iPr} **6**

Fig. 1. Mixed ligand sandwich ruthenium(II) complexes.

analogues for the synthesis of bioconjugates with possible applications in biomedical or electrochemical studies.

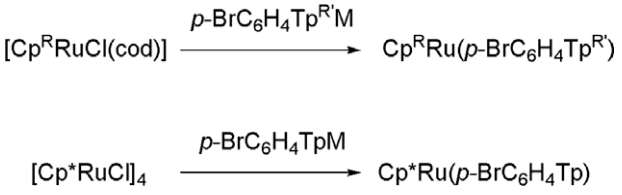
Herein, we report the synthesis and characterization of several mixed ligand Cp/Tp ruthenium compounds depicted in Fig. 1. Further, we demonstrate the conversion of $Cp^{iPr}Ru(p\text{-}BrC_6H_4Tp)$ (**6**) to the corresponding benzoic acid derivative $Cp^{iPr}Ru(p\text{-}(CO_2H)C_6H_4Tp)$ (**7**) and its coupling to a protected amino acid in solution as well as to the neuropeptide enkephalin by solid phase peptide synthesis (SPPS).

2. Results

2.1. Synthesis and characterization of the mixed ligand sandwich ruthenium complexes

Of the two basic options for the assembly of the ruthenocene analogues starting from "half-sandwich" complexes, the introduction of the Cp ligand prior to the Tp ligand was found to be the favourable method. While CpRuCl(cod) (cod = 1,5-cyclooctadiene) is kinetically labile and is known to undergo reaction with Tp transfer agents giving CpRuTp species [11,12], the Tp analogue TpRuCl(cod) is known to be quite inert to ligand substitution [20,21]. As outlined in Scheme 1, compounds **3**, **4** and **6** were obtained by modifying the parent syntheses of CpRuTp from species containing a $[RuCp^R]$ synthon [22], while **5** was synthesized starting from $\{Cp^*RuCl_4\}$ [23].

Quite notably, we found **3–6** to differ remarkably in terms of stability and solubility. For example, CpRuTp′ (**3**) was found to be poorly soluble in most organic solvents. As a result, **3** did not react



Scheme 1. Syntheses of mixed ligand sandwich ruthenium compounds, see also Fig. 1 for definitions of R and R′.

with *n*-BuLi neither in diethylether nor in THF at −78 °C and thus has not been suitable for functionalisation towards bioconjugates. Further, the low solubility prevented characterization by ^{13}C NMR within a reasonable time-period and only the ^1H spectrum was obtained. To increase the solubility of the complex we considered the use of substituted derivatives of Tp.

Reaction of $Tp'^{Me}Tl$ (**1**) with CpRuCl(cod) at room temperature gave $CpRuTp'^{Me}$ (**4**). Characterization by ^1H NMR showed rapid decomposition of samples prepared in air by colour change and shift of the signals of both ligands. Subsequent samples prepared under argon atmosphere showed similar decomposition to unidentified products after a couple of hours which made the recording of a ^{13}C NMR impossible. Due to the requirement of stability, no further investigations were made.

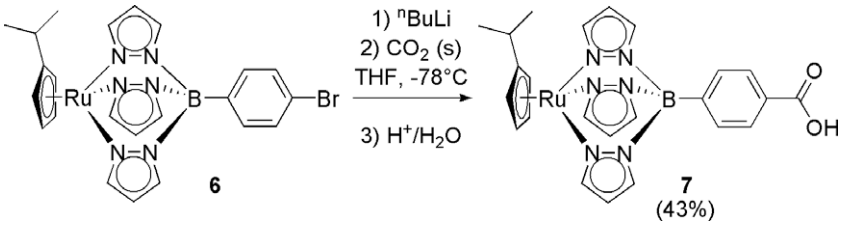
Similar observations were made with samples of Cp^*RuTp' (**5**). In this case, the decomposition proceeded rapidly even in samples prepared under argon atmosphere. Monitoring the ^1H spectra proved complete decomposition within 1 h. Low stability in solution has been reported for other Cp^*MTp (M = Ni, V, Fe) complexes as well [24], whereas related homoleptic metallocenes are known to be air-stable [25,26].

Regarding the observations that an increasing number of methyl groups raises the solubility, but seems to have negative effects on the stability of the compounds, we considered less substituted Cp ligands as an option to proceed in terms of a stable compound.

Modifying the literature preparation of CpRuCl(cod) [12], we synthesized $Cp^{iPr}RuCl(cod)$ (**2**) by substituting TlCp with $NaCp^{iPr}$ in comparable yield as a golden yellow solid. Reaction of **2** and Tp′Li gave $Cp^{iPr}Ru(p\text{-}BrC_6H_4Tp)$ (**6**). Characterization by ^1H NMR showed remarkable changes for the Cp^{iPr} ligand compared to the starting material. The formerly singlet signal of the four aromatic protons appears as a pair of *pseudo*-triplets [27]. This kind of splitting is known and has been noted recently for related $Cp^RRu(L_2X)$ complexes [28].

$Cp^{iPr}Ru(p\text{-}BrC_6H_4Tp)$ (**6**) was found to be stable to air both in solution and as a solid. Reacting **6** with *n*-BuLi in THF at −78 °C followed by addition of solid CO_2 and workup with hydrochloric acid, as depicted in Scheme 2, gave the benzoic acid-functionalized species $Cp^{iPr}Ru(p\text{-}(CO_2H)C_6H_4Tp)$ (**7**). Success of the reaction was proven by the appearance of one stretch for the COOH group at 1685 cm^{-1} in IR samples [29].

The solid state structure of **7** was determined by X-ray diffraction. The ORTEP view of the molecule is shown in Fig. 2, giving the atom labelling and selected bond lengths and angles. The structure was found to be consistent with the "piano-stool" like appearance of unsubstituted CpRuTp [11], showing local C_{3v} symmetry of the Tp′ ligand with an average Ru–N bond length of 2.113(4) Å (2.126 Å for the unsubstituted compound) and an average N–Ru–N angle of 82.9(4)°. The Ru–C bonds have an average length of 2.149(5) Å, resulting in a Ru–Cp centroid distance of 1.780(1) Å that is only slightly longer than in the unsubstituted compound CpRuTp (1.777 Å), but still in the range found for other cyclopentadienyl compounds of Ru.



Scheme 2. Introduction of the acid functionality to the mixed ligand sandwich complex **6**.

2.2. Synthesis and characterization of mixed ligand sandwich bioconjugates

In order to test the suitability of $Cp^{iPr}Ru(p\text{-}(CO_2H)C_6H_4Tp)$ (**7**) in the coupling to peptides, it was coupled to phenylalanine-methylester (H-Phe-OMe) under standard conditions in dichloromethane to give the amide $Cp^{iPr}Ru(p\text{-}(CO\text{-Phe-OMe})C_6H_4Tp)$ (**8**) as depicted in Scheme 3. Formation of the amide moiety [NHCO] is unambigously supported by ^1H NMR (d, $\delta = 6.64$; $J = 7.5$), ^{13}C NMR ($\delta = 167.0$ ppm) and IR (1653 cm^{-1}) spectroscopies [29].

Compound **7** was additionally tested in SPPS methodology by coupling to the pentapeptide Enkephalin (Enk = -Tyr-Gly-Gly-Phe-Leu) [30] as depicted in Scheme 4 [31]. Specific conditions had to be chosen concerning the low stability of **7** against TFA [32], a commonly used cleavage reagent and thus the base labile HMBA (4-hydroxymethylbenzoic acid) linker was used so as to

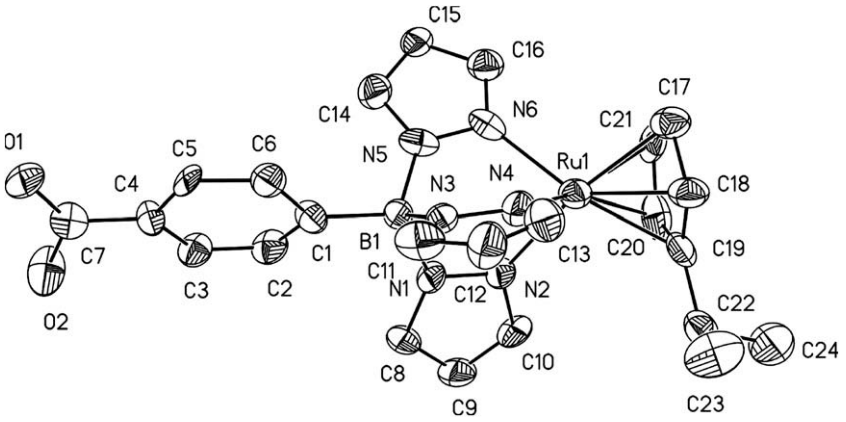
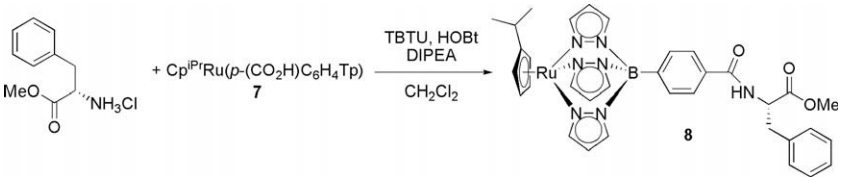
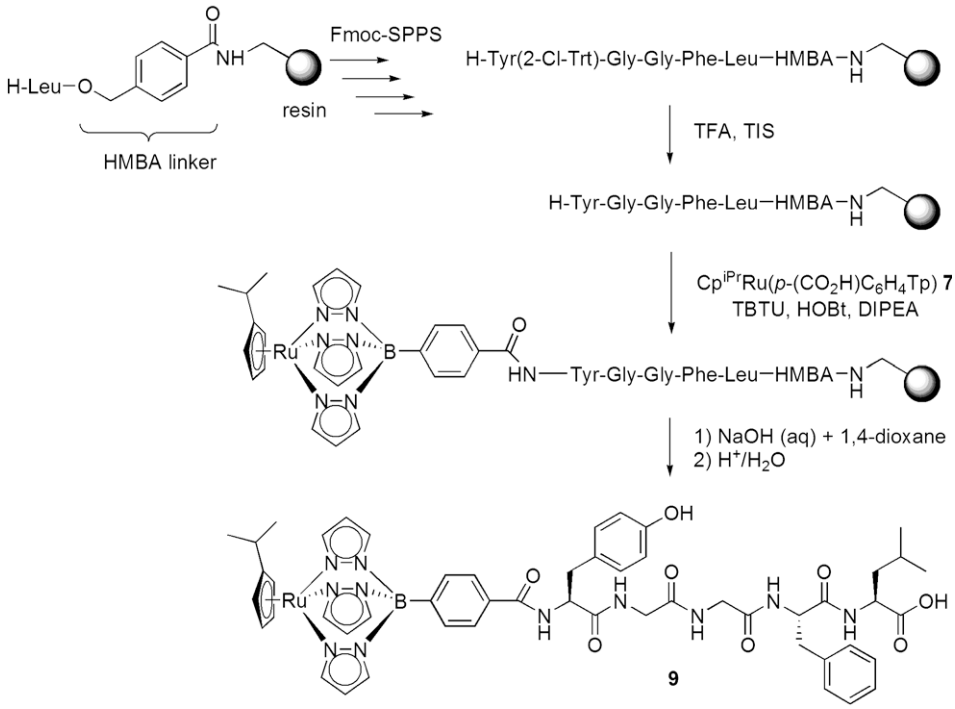


Fig. 2. ORTEP plot of the structure of $Cp^{iPr}Ru(p\text{-}(CO_2H)C_6H_4Tp)$ **7**, Hydrogens omitted. Selected bond lengths (Å): Ru1–N2: 2.123(8); Ru1–N4: 2.124(11); Ru1–N6: 2.112(9); Ru1–C17: 2.158(11); Ru1–C18: 2.155(12); Ru1–C19: 2.201(11); Ru1–C20: 2.132(12); Ru1–C21: 2.165(11) Ru1–Cp^{iPr} centroid 1.790; N–Ru–N angles are 81.9(3)–82.5(3)°.



Scheme 3. Coupling of **7** to phenylalanine-methylester.



Scheme 4. SPPS coupling of **7** to enkephalin.

avoid the need for acid treatment altogether. Enkephalin was synthesized according to standard Fmoc-SPPS methods [5]. After removal of the 2-chloro-trityl (2-Cl-Trt) protecting group from tyrosine, $Cp^{iPr}Ru(p\text{-}(CO_2H)C_6H_4Tp)$ (**7**) was coupled. While the presence of a hydroxy group might interfere in coupling of the amino acids, it has been shown before to have little influence in the coupling of metal complexes [33]. The resulting bioconjugate Cp^{iPr}-$Ru(p\text{-}(CO\text{-}Enk\text{-}OH)\text{-}C_6H_4Tp)$ (**9**) was subsequently cleaved from the resin under basic conditions. Compound **9** was purified by reverse phase-HPLC and characterized by mass and ^1H NMR spectroscopies. The electrospray ionization MS (negative mode) showed a signal centered around $m/z = 1078.3$, which corresponds exactly to the carboxylate form of **9**. The observed isotope pattern matched the calculated one, in particular clearly indicating the presence of the Ru atom. All NMR signals could be assigned with the help of literature data and standard 2D NMR spectroscopic methods. The integration of the ^1H NMR signals match with the proposed composition, e.g. same intensities were observed for the isopropyl groups on the Cp ring and of the leucine amino acid.

3. Conclusion

In summary, this work presents an entry into mixed ligand ruthenium sandwich compounds, in particular combining cyclopentadienyl and tris(pyrazolyl)borate ligands. Although several derivatives with different substituent patterns on the Cp and Tp ligands seemed to be readily accessible, only one complex **6** emerged with the appropriate combination of solubility and stability for use in bioconjugates. Prolonged stability in air and water is obviously a prerequisite for biological applications. An acid-functionalised derivative **7** of the parent compound **6** was easily available and could be successfully coupled to an amino acid in solution. Solid phase peptide synthesis (SPPS) poses even more challenges for the synthesis of bioconjugates, because additional *chemical* stability is required. In this case, the mixed ligand Ru sandwich compounds were not stable in acidic media, hence a peptide synthesis scheme (with appropriate resin, linker and side chain protection/deprotection) has been chosen which avoids the commonly used acidic conditions altogether. In this way, the first mixed ligand sandwich (Cp/Tp) ruthenium peptide bioconjugate could finally be obtained and was fully characterised. Ruthenium-103 derivatives of estrogens and amino acids have been used for radiopharmaceutical applications in early work by Wenzel and coworkers [34]. There is an obvious structural similarity between ruthenocene and ferrocene, which is by far the most widely used metallocene in bioconjugates [4h–k]. Notably however, ruthenocene has only recently been used to label peptides in a solid phase synthesis scheme [35]. The mixed ligand CpRuTp′ compounds used in this work not only further extend the range of Ru complexes for bioconjugates by replacing one Cp ligand in ruthenocene with a substituted tris(pyrazolyl)borate ligand. Also, it shows the broad range and universal applicability of possible Tp complexes for biological applications. Further work on metal-Tp bioconjugates and their biological activity is in progress in our laboratory.

4. Experimental

4.1. General remarks

Unless noted otherwise, all preparations were carried out under an inert atmosphere of argon or N_2 using standard Schlenk techniques and a M-Braun glovebox. All reagents and anhydrous solvents were purchased from commercial sources and used as received. Tentagel-S resin with a HMBA linker was obtained from NovaBiochem, as were the protected amino acid building blocks. Enantiomerically pure L amino acids were used throughout. 1-Hydroxy-1H-benzotriazole (HOBt) and benzotriazol-1-yl-*N*-tetramethyl-uroniumtetrafluoroborate (TBTU) were purchased from Iris Biotech (Germany). The reagents $p\text{-}BrC_6H_4BBr_2$, $(p\text{-}BrC_6H_4)TpLi$ [8,9,36], $[Ru(H)(cod)(NH_2NMe_2)_3][PF_6]$ [37], CpRuCl(cod) [12] and $\{Cp^*RuCl\}_4$ [38] were prepared by literature procedures. NMR spectra were recorded at ambient temperature on Bruker DPX 200, DPX 250 and DRX 600 spectrometers. The chemical shifts (δ) are reported in parts-per-million (ppm) relative to the residual proton chemical shifts of the deuterated solvent set relative to external TMS. Coupling constants (J) are quoted in Hertz. $^{13}C\{^1H\}$ assignments were obtained from standard attached proton test (APT) and heteronuclear single quantum coherence (HSQC) experiments. IR spectra were recorded on a Bruker Tensor 27 spectrometer with an ATR unit as solid samples, wavelengths of absorption are given in cm^{-1}. Electrospray ionisation mass spectra (ESI-MS) were recorded on a Bruker Esquire 6000 spectrometer. The analytical and preparative HPLC were both carried out on a Varian Prostar instrument using a RP Varian Dynamax analytical column (C18 microsorb 60 Å, diameter 4.5 mm, length 250 mm). Eluents were water and acetonitrile both containing 0.1% v/v TFA using a linear gradient of 15–100% acetonitrile for 30 min at a flow rate of 1 ml/min. Elemental analyses of ruthenium containing compounds were carried out at the laboratory for microanalytics and thermal analyses, University of Essen (Inorganic Chemistry Department), all others were carried out at the RUBiospek Biospectroscopy Department, Ruhr-Universität Bochum.

4.1.1. $(p\text{-}BrC_6H_4)Tp^{Me}Tl$ (**1**)

A solution of dibromo-(4-bromophenyl)borane (2.00 g, 6.12 mmol) in dichloromethane (10 mL) was added over 60 min to a cooled (0 °C) and rapidly stirred solution of 3-methylpyrazole (1.56 g, 18.97 mmol) and triethylamine (2.66 mL, 21.64 mmol) in dichloromethane (4 mL). After stirring for 12 h at room temperature, the solvent was removed and the remaining white solid was extracted with THF (2×15 mL). Thalliumethoxide (1.85 g, 6.98 mmol) was added to the extract. After stirring for 12 h, the solvent was removed to give a colourless solid, which was washed with diethylether (2×10 mL). Drying under lowered pressure yielded the product as a colourless solid (2.05 g, 3.34 mmol, 55%). Anal. Calc. for $C_{18}H_{19}BBrN_6Tl$: C, 35.18; H, 3.12; N, 13.68. Found C, 35.81; H, 3.57; N, 13.03%. ^1H NMR ($CDCl_3$) δ 7.51 (d, $J = 8.4$, 2H), 7.31 (d, $J = 8.4$, 2H), 7.25 (d, $J = 2.0$, 3H), 5.99 (d, $J = 2.0$, 3H), 2.36 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 149.6 (CH), 137.1 (CH), 136.5 (CH), 130.9 (C-B), 122.3 (C-Br), 104.9 (CH), 13.7 (CH_3).

4.1.2. $Cp^{iPr}RuCl(cod)$ (**2**)

A solution of $[Ru(H)(cod)(NH_2NMe_2)_3][PF_6]$ (2.14 g, 4.0 mmol) and $NaCp^{iPr}$ (0.57 g, 4.4 mmol) in THF (30 mL) was heated under reflux for 45 min. Removal of the volatiles gave a brown sticky solid which was extracted with *n*-hexane (2×35 mL). Dropwise addition of tetrachloromethane (0.5 mL, 4.0 mmol) to the extract produced an orange precipitate, which was separated by filtration at 0 °C and dried under lowered pressure to yield a golden orange solid (1.20 g, 3.41 mmol, 85%). Anal. Calc. for $C_{16}H_{23}ClRu$: C, 54.61; H, 6.59. Found C, 54,76; H, 6.63%. ^1H NMR ($CDCl_3$) δ 5.14 (m, 2H), 4.78 (s, 4H), 4.26 (m, 2H), 2.63–2.54 (m, 3H), 2.07 (m, 4H), 1.97 (m, 2H), 1.08 (d, $J = 6.9$, 6H); ^{13}C NMR ($CDCl_3$) δ 88.3 (CH), 86.2 (CH), 80.7 (C), 78.7 (CH), 32.4 (CH_2), 28.1 (CH_2), 25.8 ($CH(CH_3)_2$), 22.7 (CH_3).

4.1.3. $CpRu(p\text{-}BrC_6H_4Tp)$ (**3**)

CpRuCl(cod) (0.11 g, 0.36 mmol) and $p\text{-}BrC_6H_4TpLi$ (0.15 g, 0.39 mmol) were mixed and dissolved in THF (10 mL) and the solution was stirred for 2 h. The solvent was removed under lowered pressure and the residue was extracted with boiling chloroform

(50 mL). Filtration of the light yellow extract followed by removal of the solvent yielded a pale yellow solid (85.5 mg, 0.16 mmol, 45%). ^{1}H NMR ($CDCl_3$) δ 8.11 (dd, J = 2.2; 0.5, 3H), 7.75 (d, J = 8.4, 2H), 7.63 (d, J = 8.4, 2H), 7.40 (d, J = 2.2; 0.5, 3H), 6.13 (t, J = 2.2, 3H), 4.30 (s, 5H).

4.1.4. CpRu(p-BrC$_6$H$_4$Tp^{Me}) (**4**)

CpRuCl(cod) (0.17 g, 0.55 mmol) and p-BrC$_6$H$_4$Tp^{Me}Tl **1** (0.29 g, 0.46 mmol) were mixed and dissolved in THF (10 mL) Stirring for 1 h gave a pale green solution, that was filtered. The remaining insolubles were extracted with THF (3 × 5 mL). Removal of the solvent from the combined extracts and filtrate gave the product as a yellow solid (0.12 g, 0,21 mmol, 45%). ^{1}H NMR ($CDCl_3$) δ 7.69 (d, J = 8.4, 2H), 7.56 (d, J = 8.4, 2H), 7.28 (d, J = 2.2, 3H), 5.93 (d, J = 2.2, 3H), 4.71 (s, 5H), 2.50 (s, 9H); IR (solid): 2970 ν(Ar-H), 1573 ν(C=N); ESI-MS (pos.): m/z = 577.00 [M+H]$^+$, exact mass of $C_{23}H_{24}BBrN_6Ru$ = 576.04.

4.1.5. Cp*Ru(p-BrC$_6$H$_4$Tp) (**5**)

THF (4 mL) was slowly added to a mixture of {Cp*RuCl$_4$} (0.31 g, 1.15 mmol) and p-BrC$_6$H$_4$TpLi (0.43 g, 1.15 mmol). The resulting orange solution was stirred at room temperature for 2 h. Upon removal of the solvent, a yellow solid precipitated from the solution. It was isolated by filtration, washed with diethylether (2 × 15 mL) and dried under lowered pressure to give a yellow solid (0.21 g, 0.35 mmol, 30%).^{1}H NMR (C_6H_6) δ 8.07 (dd, J = 2.2; 0.5, 3H), 7.52 (d, J = 8.4, 2H), 7.32 (d, J = 8.4, 2H), 7.27 (dd, J = 2.2; 0.5, 3H), 5.97 (t, J = 2.2, 3H), 1.59 (s, 15H); IR (solid): 2962 ν(Ar-H), 1580 ν(C=N); ESI-MS (pos.): m/z = 605.05 [M+H]$^+$, exact mass of $C_{25}H_{28}BBrN_6Ru$ = 604.07.

4.1.6. Cp^{iPr}Ru(p-BrC$_6$H$_4$Tp) (**6**)

Cp^{iPr}RuCl(cod) (**2**) (0.19 g, 0.53 mmol) and p-BrC$_6$H$_4$TpLi (0.23 g, 0.60 mmol) were mixed and dissolved in THF (15 mL). The solution was stirred for 45 min at room temperature and filtered to give a clear light green solution which was reduced to about 5 mL under lowered pressure. Cooling the concentrated solution to −20 °C overnight yielded a yellow precipitate. It was isolated by filtration and dried under lowered pressure to give a yellow microcrystalline powder (0.23 g, 0.40 mmol, 76%). Anal. Calc. for $C_{23}H_{24}BBrN_6Ru$: C, 47.94; H, 4.20; N, 14.58. Found: C, 47.95; H, 4.74; N, 14.82%. ^{1}H NMR ($CDCl_3$) δ 8.09 (dd, 3H, J = 2.2; 0.5), 7.77 (d, J = 8.4, 2H), 7.61 (d, J = 8.4, 2H), 7.39 (dd, J = 2.2; 0.5, 3H), 6.12 (t, J = 2.2, 3H), 4.30 (t, J = 1.6, 2H), 4,06 (t, J = 1.6, 2H), 2.60 (sept., 1H, J = 6.8), 1.18 (d, 6H, J = 6.8); ^{1}H NMR ($CDCl_3$) δ 145.0 (CH), 136.5 (CH), 134.9 (C-B), 136.5 (CH), 134.0 (CH), 127.9 (C-Br), 105.1 (CH), 85.5 (C_q), 73.1 (CH), 64.8 (CH), 26.7 (CH(CH$_3$)$_2$), 24.1 (CH$_3$); ESI-MS (pos.): m/z = 432 ([M−C$_6$H$_4$Br+H]$^+$), 498 ([M−Cp^{iPr}+Na]$^+$), 577 ([M+H]$^+$), exact mass for $C_{23}H_{24}BBrN_6Ru$ = 576.04.

4.1.7. Cp^{iPr}Ru(p-(CO$_2$H)C$_6$H$_4$Tp) (**7**)

Cp^{iPr}Ru(p-BrC$_6$H$_4$Tp) (**6**) (0.10 g, 0.17 mmol) was dissolved in THF (25 mL) and the solution was cooled to −70 °C. n-BuLi (0.12 mL, 1.6 M in hexane, 0.19 mmol) was added dropwise and the resulting mixture was stirred for 1 h. After addition of excess solid CO_2 (~5 g), the solution was allowed to warm to room temperature. The volatile components were removed under lowered pressure to obtain a pale yellow solid. On the benchtop open to air, it was slurried in water (5 mL) and treated with hydrochloric acid (2 mL, 2 N) to give a yellow precipitate. It was washed with water (2 × 10 ml) and dried under lowered pressure to give a bright yellow solid (38 mg, 0.07 mmol, 43%). Anal. Calc. for $C_{24}H_{25}BN_6O_2Ru$: C, 53.25; H, 4.65; N, 15.52. Found: C, 53.23; H: 4.80; N, 15.09%. ^{1}H NMR (DMSO-d_6) δ 12.9 (s, 1H), 8.26 (d, J = 2.2, 3H), 8.07 (d, J = 8.4, 2H), 7.95 (d, J = 8.4, 2H), 7.37 (d, J = 2.2, 3H), 6.19 (t, J = 2.2, 3H), 4.54 (t, 2H), 4.13 (t, 2H), 2.57 (sept., J = 6.8, 1H), 1.11 (d, J = 6.8, 6H); ^{13}C NMR (DMSO-d_6) δ 167.4 (CO_2H), 145.2 (CH), 136.8 (C-B), 134.6 (CH), 133.7 (CH), 128.6 (CH), 128.0 (C-CO_2H), 105.4 (CH), 84.3 (C_q), 73.6 (CH), 64.7 (CH), 25.9 (CH(CH$_3$)$_2$), 23.9 (CH$_3$). IR (solid) 1685; ESI-MS (pos.): m/z = 542.07 [M+H]$^+$, exact mass for $C_{24}H_{25}BN_6O_2Ru$ = 541.38.

X-ray structure determination of **7**: A crystal of **7** (yellow needle), obtained by slow evaporation of a CH_2Cl_2 solution, was placed on a glass capillary in perfluorinated oil and measured in a cold gas flow. The intensity data were measured with a Bruker axs area detector (Mo Kα radiation 0.71073 Å, ω scan) at −60 °C. $C_{24}H_{25}BN_6O_2Ru$ M = 542.12, triclinic, a = 9.359(5) Å, b = 13.557(7) Å, c = 20.31(1) Å, α = 88.06(1)°, β = 78.83(1)°, γ = 79.54(1)°, V = 2487(2) Å^3, space group $P\bar{1}$, Z = 2, 11524 reflections collected, 8341 unique (R_{int} = 0.0734), $wR_2(F^2)$ = 0.2420 (all data). Bruker-axs-SMART 1000 CCD. Structure solution with direct methods (SHELXS97 [39]), and refined against F^2 with all measured reflections (SHELXL97 [39] and Platon/Squeeze [40]). CCDC 699278 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

4.1.8. Cp^{iPr}Ru(p-(CO-Phe-OMe)C$_6$H$_4$Tp) (**8**)

Cp^{iPr}Ru(p-(CO$_2$H)C$_6$H$_4$Tp) (**7**) (190 mg, 0.35 mmol) and TBTU (112 mg, 0.35 mmol) were mixed in dichloromethane (12 mL) and DIPEA (0.41 mL, 2.45 mmol) was added to the slurry. After 10 min, phenylalanine-methylester hydrochloride (76 mg, 0.35 mmol) was added and the resulting solution was stirred over night. Removal of the solvent gave a brown oily residue, that was extracted into a minimum amount of dichloromethane. The solution was eluted from a silica column [60 Å, CH_2Cl_2] to give the product as a yellow solid (130 mg 0.19 mmol, 53%) after removal of the solvent. Anal. Calc. for $C_{34}H_{36}BN_7O_3Ru$: C, 58.12; H, 5.16; N, 13.96. Found: C, 58.11; H, 5.34; N, 13.87%. ^{1}H NMR ($CDCl_3$) δ 8.10 (d, J = 2.2, 3H), 7.98 (d, J = 8.4, 2H), 7.83 (d, J = 8.4, 2H), 7.39 (d, J = 2.2, 3H), 7.33–7.16 (m, 5H), 6.64 (d, J = 7.5, 1H), 6.12 (t, J = 2.2, 3H), 5.16 (m, 1H), 4.31 (t, J = 1.6, 2H), 4.06 (t, J = 1.6, 2H), 3.30 (m, 2H), 2.61 (sept., J = 6.8, 1H), 1.18 (d, J = 6.8, 6H); ^{13}C NMR ($CDCl_3$) δ 171.5 (CO_2Me), 167.0 (CONH), 147.7 (CH), 140.0 (C_q), 135.2 (C-B), 134.2 (CH), 129.5–126.4 (CH), 105.3 (CH), 85.4 (C_q), 73.1 (CH), 64.8 (CH), 53.7 (CH), 52.6 (OCH_3), 38.1 (CH_2), 26.6 (CH(CH$_3$)$_2$), 24.3 (CH$_3$). ESI-MS (pos.): m/z = 703.09 ([M+H]$^+$ exact mass for $C_{34}H_{36}BN_7O_3Ru$ = 702.58), 443.19 ([Cp^{iPr}Ru(pz)$_3$+Na]$^+$). IR (solid): 1740, 1653 cm^{-1}.

4.1.9. Cp^{iPr}Ru(p-(CO-Tyr-Gly-Gly-Phe-Leu-CO$_2$H)C$_6$H$_4$Tp) (**9**)

Resin-bound enkephalin Tyr(2-Cl-Trt)-Gly-Gly-Phe-Leu(HMBA-RES) was obtained by standard Fmoc-SPPS starting from Fmoc-Leu loaded Tentagel-S resin with a HMBA linker (500 mg, load 0.24 mmol/g). After 2-Cl-Trt deprotection of the tyrosine hydroxy group with 5% TFA v/v/5% TIS v/v in CH_2Cl_2 the metal complex **7** was coupled to the peptide as described here: Cp^{iPr}Ru(p-(CO$_2$H)C$_6$H$_4$Tp) (**7**) (140 mg, 0.23 mmol), TBTU (150 mg, 0.47 mmol), HOBt (65 mg, 0.48 mmol) and DIPEA (121 μL, 0.72 mmol) were mixed in DMF (3 mL) and stirred for 5 min. The homogenous solution was then added to the resin-bound peptide and the mixture was shaken for ca. 20 h. After filtering the reaction mixture, the resin-bound product was washed with DMF (5 × 2 mL) and dichloromethane (5 × 2 mL) and dried under reduced pressure for 1 h. The bioconjugate was cleaved from the resin by shaking it with a cooled (0 °C) mixture of aqueous NaOH solution (2.5 mL, 1M) and 1,4-dioxane (7.5 mL) for 10 min. The resulting yellow solution was filtered and adjusted to pH 7 by addition of dilute hydrochloric acid. Removal of the solvents under lowered pressure gave a mixture of colourless NaCl and a dark yellow solid. It was taken into a minimum volume of methanol,

filtered from NaCl and purified by reversed phase-HPLC to give a yellow solid (80 mg, 0.07 mmol, 62% based on resin load).

^{1}H NMR (CD_3OD) note: The resonances from one α-CH and two β-CH_2 groups are obscured by the solvent peaks. Assignments are based, in part, upon comparison to literature data [41]. δ 8.04 (d, J = 8.0, 2H, CH of B-C_6H_4), 7.96 (d, J = 8.0, 2H, CH of B-C_6H_4), 7.84 (d, J = 2.2, 3H, CH of pz), 7.63 (d, J = 2.2, 3H, CH of pz), 7.26–7.14 (m, 5H, C_6H_5 of Phe), 7.15 (d, J = 8.0, 2H, CH of Tyr *p*-phenol), 6.75 (d, J = 8.0, 2H, CH of Tyr *p*-phenol), 6.32 (d, J = 2.2, 3H, CH of pz), 4.66 (dd, J = 9.0, 5.5, 1H, α-CH), 4.62 (s, 2H, CH of CpiPr), 4.47 (s, 2H, CH of CpiPr), 4.40 (dd, J = 9.0, 5.5, 1H, α-CH), 3.86 (m, 4H, α-CH_2 of Gly), 3.24 (dd, J = 13.8, 9.0, 2H, β-CH_2), 2.97 (dd, J = 13.8, 9.0, 2H, β-CH_2), 1.70–1.58 (m, 4H, α-CH, β-CH_2, $CH(CH_3)_2$ of Cp^{iPr}), 1.19 (d, J = 6.3 Hz, 6H, CH_3 of Cp^{iPr}), 0.88 (d, J = 6.3, 3H, Leu-CH_3), 0.83 (d, J = 6.3, 3H, Leu-CH_3). ESI-MS (neg.): m/z = 1078.29 $[M-H]^-$, exact mass for $C_{52}H_{60}BN_{11}O_8Ru$ = 1079.38.

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