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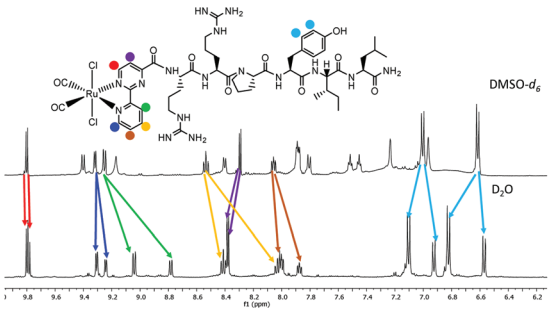
Ru(II)-Peptide bioconjugates with the cppH linker (cppH = 2-(2′-pyridyl)pyrimidine-4-carboxylic acid): synthesis, structural characterization, and different stereochemical features between organic and aqueous solvents

Federica Battistin, Daniel Siegmund, Gabriele Balducci, Enzo Alessio* and Nils Metzler-Nolte*

Three new Ru(II)-peptide bioconjugates were prepared in pure form and fully characterized. Unlike in organic solvents, stable stereoisomers were found by NMR studies in D_2O.



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First (given) and middle name(s)	Last (family) name(s)	ResearcherID	ORCID iD
Federica	Battistin		
Daniel	Siegmund		0000-0003-2476-8965
Gabriele	Balducci		0000-0002-0007-0880
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Ru(II)-Peptide bioconjugates with the cppH linker (cppH = 2-(2′-pyridyl)pyrimidine-4-carboxylic acid): synthesis, structural characterization, and different stereochemical features between organic and aqueous solvents†

Federica Battistin,[a] Daniel Siegmund,[b] Gabriele Balducci,[a] Enzo Alessio *[a] and Nils Metzler-Nolte *[b]

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Three new Ru(II) bioconjugates with the C-terminal hexapeptide sequence of neurotensin, RRPYIL, namely *trans,cis*-$RuCl_2(CO)_2$(cppH-RRPYIL-κN^P) (**7**), [Ru([9]aneS$_3$)(cppH-RRPYIL-κN^P)(PTA)]$(Cl)_2$ (**8**), and [Ru([9]aneS$_3$)Cl(cppH-RRPYIL-κN^P)]Cl (**11**), where cppH is the asymmetric linker 2-(2′-pyridyl)pyrimidine-4-carboxylic acid, were prepared in pure form and structurally characterized in solution. The cppH linker is capable of forming stereoisomers (*i.e.* linkage isomers), depending on whether the nitrogen atom *ortho* (N^O) or *para* (N^P) to the carboxylate on C4 in the pyrimidine ring binds the metal ion. Thus, one of the aims of this work was to obtain pairs of stereoisomeric conjugates and investigate their biological (anticancer, antibacterial) activity. A thorough NMR characterization clearly indicated that in all cases exclusively N^P conjugates were obtained in pure form. In addition, the NMR studies showed that, whereas in DMSO-d_6 each conjugate exists as a single species, in D_2O two (**7**) or even three if not four (**8** and **11**) very similar stable species form (each one corresponding to an individual compound). Similar results were observed for the cppH-RRPYIL ligand alone. Overall, the NMR findings are consistent with the occurrence of a strong intramolecular stacking interaction between the phenol ring of tyrosine and the pyridyl ring of cppH. Such stacking interactions between aromatic rings are expected to be stronger in water. This interaction leads to two stereoisomeric species in the free cppH-RRPYIL ligand and in the bioconjugate **7**, and is somehow modulated by the less symmetrical Ru coordination environments in **8** and **11**, affording three to four very similar species.

Introduction

The synthesis and biological evaluation of metal bioconjugates, in which a targeting molecule binds one or more metal fragments, is a very active field of bioinorganic chemistry, with applications in radiolabeling, biosensing, as well as anticancer and antimicrobial drug development.[1] Receptor-binding peptides, such as neurotensin (NT) and octreotide (an analogue of somatostatin) are among the most actively investigated biomolecules. They are characterized by a high affinity for receptors that are overexpressed in several tumors, and thus they have the potential to internalize selectively the bound metal into target cancer cells in the so-called "trojan horse approach".[2,3]

In this work we focused on neurotensin (NT), a tridecapeptide hormone with primary structure pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu. Three receptors (NTR1–3) recognize the C-terminal hexapeptide sequence of NT, Arg-Arg-Pro-Tyr-Ile-Leu or RRPYIL (or $NT_{8–13}$), which is thus sufficient for recognition and binding (Fig. 1).

Since both NTR1 and NTR3 are upregulated in several human cancers such as colon, pancreatic, prostate, and lung cancer,[4] metal-bioconjugates with this peptide, or with the RRPYIL fragment, are interesting candidates for antiprolifera-

[a]*Department of Chemical and Pharmaceutical Sciences, University of Trieste, Via Giorgieri 1, 34127 Trieste, Italy. E-mail: alessi@units.it; http://www.dscf.units.it/en*
[b]*Inorganic Chemistry I – Bioinorganic Chemistry, Ruhr University Bochum, Faculty of Chemistry and Biochemistry, Bochum, Germany. E-mail: nils.metzler-nolte@rub.de; http://www.chemie.rub.de/ac1*

†Electronic supplementary information (ESI) available: Crystal and refinement data, and selected coordination distances and angles for compound **10** (Tables S1 and S2); minimal inhibitory concentrations (MIC, μg mL^{-1}) for compounds **1–4**, **7**, **8** and **11** on Gram-positive and Gram-negative bacterial strains (Table S3); additional 1D and 2D NMR spectra for compounds **7**, **8**, **10**, **11**, and cppH-RRPYIL. CCDC 1863829 (**10**). For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c8dt03575j

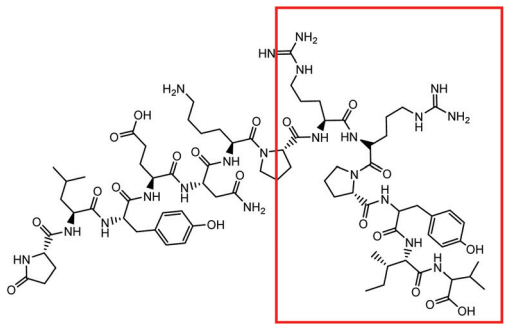


Fig. 1 Schematic representation of neurotensin; the red box indicates the C-terminal hexapeptide sequence (fragment 8–13).

tive applications. In the literature there are some examples of metal-NT_{8-13} bioconjugates, in which the hexapeptide is bound – through a peptidic bond – to a supporting ligand of the metal fragment (*e.g.* $FeCp_2$ or $RuCp_2$,[5,6] $Co_2(CO)_8$,[5] Re(I),[7] Pt(IV)[8]). In one case the peptide is connected to the Rh(III)Cp* fragment through the Tyr phenol ring.[9]

In general, the preparation of metal-bioconjugates requires the use of a bifunctional linker, *i.e.* a molecule that is capable of binding firmly the metal ion (typically forming a chelate ring, for thermodynamic and kinetic stability) and has another functionality for binding the biomolecule through a covalent bond. The asymmetric chelating diimine 2-(2′-pyridyl)pyrimidine-4-carboxylic acid (cppH, Fig. 2) was introduced as a new linker by Spiccia and coworkers in 2009.[10] In contrast with the similar 4′-methyl-2,2′-bipyridine-4-carboxylic acid, cppH has a straightforward preparation but is capable of forming stereoisomers (*i.e.* linkage isomers): in fact, its pyrimidine ring can bind to the metal ion either through the nitrogen atom *ortho* (N^o) or *para* (N^p) to the carboxylate on C4. This potential disadvantage can, on the other hand, be exploited for preparing stereoisomeric conjugates. The cppH linker has been used in the preparation of Ru(II)-bioconjugates with PNA and peptides for biosensing and biomedical applications; in all the reported examples it was always bonded through N^p.[11,12]

In recent years we have described the preparation in pure form and the full characterization of both linkage isomers of two Ru(II)-cppH complexes, namely *trans*,*cis*-$RuCl_2(CO)_2$(cppH-κN^p) (**1**) and *trans*,*cis*-$RuCl_2(CO)_2$(cppH-κN^o) (**2**) (obtained by reaction of cppH with the precursor *trans*,*cis*,*cis*-$RuCl_2(CO)_2(dmso\text{-}O)_2$ (**3**)), and [Ru([9]aneS$_3$)(cppH-κN^p)(PTA)](Cl)$_2$ (**4**) and [Ru([9]aneS$_3$)(cppH-κN^o)(PTA)](Cl)$_2$ (**5**) (obtained by reaction of cppH with the precursor Ru([9]aneS$_3$)Cl$_2$(PTA) (**6**), PTA = 1,3,5-triaza-7-phosphaadamantane) (Fig. 2).[13,14]

We argued that such pairs of linkage isomers might offer – in principle – the unique opportunity to prepare unprecedented stereoisomeric Ru-bioconjugates that differ only in the orientation of the macromolecule with respect to the metal fragment, and to investigate if they behave differently in terms of activity/selectivity in biological experiments.

In this paper we describe the preparation in pure form and characterization of three novel Ru(II)-peptide bioconjugates, obtained by linking the cppH carboxylic group to the C-terminal arginine of the hexapeptide sequence of neurotensin, RRPYIL, through a peptide bond. Since the asymmetric ligand cppH can generate – besides linkage isomers – also stereoisomers, highly symmetrical Ru(II) precursors were used for eliminating this possibility. A detailed NMR investigation performed in DMSO-d_6 and D_2O, besides allowing us to establish the cppH coordination mode, afforded unexpected findings attributed to the establishment in aqueous solution of strong intramolecular stacking interactions between the linker and the peptide chain. Cytotoxicity assays against three different cell lines, colon adenocarcinoma (HT-29), breast cancer (MCF-7) and pancreatic cancer (PT-45), as well as antibacterial tests against both Gram-positive and Gram-negative strains were performed on both Ru-cppH model complexes and the bioconjugates.

Results and discussion

Treatment of complex **2** (N^o isomer) with RRPYIL, adapting a previously established SPPS peptide coupling procedure,[12] afforded bioconjugate **7** (Scheme 1). After preparative HPLC purification, analytical HPLC showed a single peak, suggesting that the reaction product was in pure form. In the ESI MS spectrum the presence of the molecular peak at 1227.3 m/z ($[M + H]^+$) was consistent with that expected for $RuCl_2(CO)_2$(cppH-RRPYIL), with m/z = 1226.6. At this stage, however, it was impossible to establish the binding mode of the cppH linker in **7** (assuming that the geometry of the metal fragment was unaffected by the coupling). In addition, since the HPLC elution time of the conjugate is mainly dictated by the peptide fragment, the possibility that compound **7** is actually a mixture of the two possible linkage isomers could not be excluded, in case they have undistinguishable retention times.

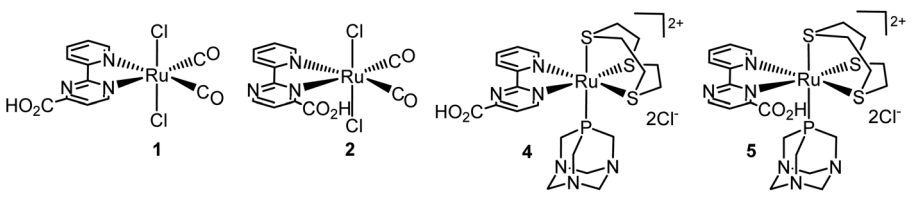
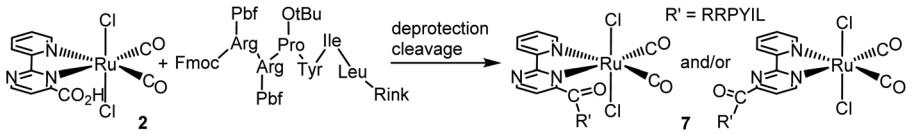


Fig. 2 The two pairs of Ru-cppH stereoisomers (linkage isomers) *trans*,*cis*-$RuCl_2(CO)_2$(cppH-κN^p) (**1**) and *trans*,*cis*-$RuCl_2(CO)_2$(cppH-κN^o) (**2**), [Ru([9]aneS$_3$)(cppH-κN^p)(PTA)](Cl)$_2$ (**4**) and [Ru([9]aneS$_3$)(cppH-κN^o)(PTA)](Cl)$_2$ (**5**).



Scheme 1 The final step of the synthesis of **7** using SPPS.

The N^p isomer **1** behaved differently and none of the mixtures of coupling agents tried for different reaction times (from 1 to 7 days), either at room temperature or in a microwave reactor (60 or 80 °C), afforded a product in acceptable yield.

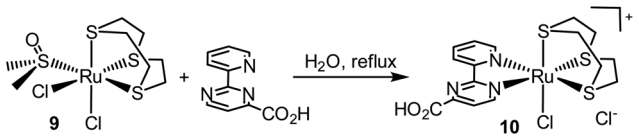
Given these results, the complementary synthetic strategy was explored. The hexapeptide RRPYIL was linked to cppH through a peptidic bond between the carboxylic group of cppH and the amino group of the terminal arginine using SPPS. The cppH-RRPYIL moiety was deprotected and cleaved from the resin, and it was then reacted with the Ru(II) precursor *trans, cis,cis*-$RuCl_2(CO)_2$(dmso-O)$_2$ (**3**) in aqueous solution at room temperature (Scheme 2), *i.e.* under the conditions that, with cppH alone, lead to a mixture of the two linkage isomers **1** and **2**.[13,15] This treatment afforded a bioconjugate whose ESI MS and analytical HPLC signatures were coincident with those of **7** described above. Thus, within the above mentioned limits, the two synthetic pathways described in Schemes 1 and 2 led to the same product **7**. Firm proof for the exact coordination mode of cppH to the ruthenium center was obtained through a thorough NMR characterization of **7**, which is reported below.

The peptide-functionalized cppH was reacted also with the precursor Ru([9]aneS$_3$)Cl$_2$(PTA) (**6**), whose reactivity towards cppH is known. In **6**, the face-capping tridentate ligand [9]aneS$_3$ gives a geometrical restrain that reduces the number of possible stereoisomers with asymmetric ligands such as cppH.[14]

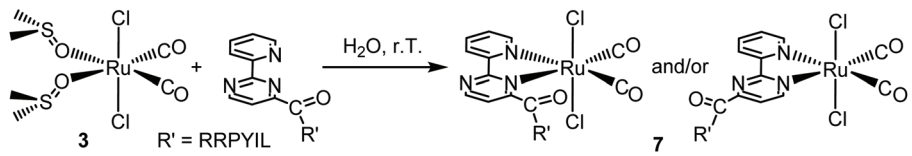
The treatment of **6** with cppH-RRPYIL, performed in water at 80 °C (*i.e.* conditions similar to those that with cppH afforded a mixture of the linkage isomers **4** and **5**),[14] yielded the bioconjugate **8** (Scheme 3). Compound **8** was pure according to analytical HPLC, and its molecular peak in the ESI MS spectrum (1438.6 *m*/*z*) was consistent with that expected for the complex cation [Ru([9]aneS$_3$)(cppH-RRPYIL)(PTA)]$^{2+}$ ([M − H$^+$]$^+$ at 1438.4 *m*/*z*).

A third bioconjugate was obtained from the Ru(II) precursor Ru([9]aneS$_3$)Cl$_2$(dmso-S) (**9**). It is known from the literature that treatment of **9** with a chelating diimine ligands such as 2,2′-bipyridine (bpy) in refluxing ethanol leads selectively to [Ru([9]aneS$_3$)Cl(bpy)]Cl.[16,17] Similarly, we found that [Ru([9]aneS$_3$)Cl(cppH)]Cl (**10**) precipitates spontaneously when **9** is reacted with cppH in refluxing water (Scheme 4). Compound **10** was fully characterized by NMR spectroscopy, mass spectrometry, and its single-crystal X-ray structure was also determined (Fig. 3).

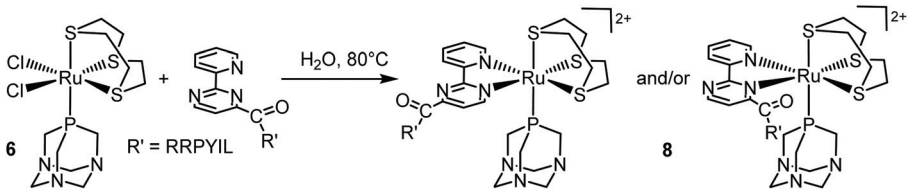
The single crystal X-ray analysis established that cppH is bound *via* N^p, *i.e.* **10** is [Ru([9]aneS$_3$)Cl(cppH-κN^p)]Cl. The ^{1}H NMR spectrum in D_2O shows two sets of six resonances for cppH, due to the equilibrium with the aqua species [Ru([9]aneS$_3$)(cppH-κN^p)(OH$_2$)]$^+$ (**10aq**) upon chloride hydro-



Scheme 4 Synthesis of the model complex [Ru([9]aneS$_3$)Cl(cppH-κN^p)]Cl (**10**).



Scheme 2 The alternative synthesis of **7**.



Scheme 3 Synthesis of bioconjugate **8**.

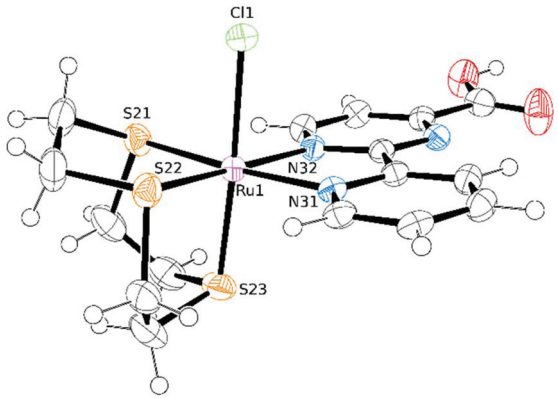


Fig. 3 X-ray molecular structure (50% probability ellipsoids) of [Ru([9]aneS$_3$)Cl(cppH-κN^p)]Cl·0.45H$_2$O (**10**). The chloride counterion and the crystallization water molecule are omitted for clarity. Coordination distances (Å): Ru1–Cl1 = 2.4316(7), Ru1–N31 = 2.094(3), Ru1–N32 = 2.096 (2), Ru1–S21 = 2.305(1), Ru1–S22 = 2.2943(8), Ru1–S23 = 2.2787(8).

lysis. The aromatic resonances of **10aq** are slightly downfield shifted (*ca.* 0.1 ppm) compared to those of the parent complex **10**, and their relative intensity decreases upon adding an excess of NaCl (ESI). The cppH chemical shifts are consistent with the N^p coordination found in the solid state (see below). The formation of the N^o isomer of **10** was not observed: the same linkage isomer was obtained also when the reaction between **9** and cppH was performed at room temperature.

The reaction between **9** and cppH-RRPYIL, performed in water at 80 °C, afforded the bioconjugate **11** (Scheme 5). After HPLC purification, the ESI MS molecular peak found for **11** (13 158 *m*/*z*) was in agreement with that expected for the cationic species [Ru([9]aneS$_3$)Cl(cppH-RRPYIL)]$^+$ (1315.1 *m*/*z*).

Also for bioconjugates **8** and **11**, as for **7** above, it was not possible, at this stage, to assess the binding mode of cppH or even whether each product was actually pure or a mixture of the two possible linkage isomers (always assuming that their biophysical properties were dominated by the peptide, and they thus might have undistinguishable retention times at HPLC analysis).

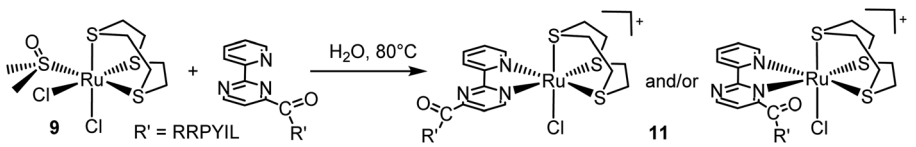
With the aim of determining the coordination mode of the cppH-RRPYIL unit, the bioconjugates **7**, **8** and **11** were subjected to a detailed analysis by NMR spectroscopy (including 2D COSY and HSQC spectra). Spectra were recorded in DMSO-d_6 and D_2O, where all compounds are completely soluble at NMR concentrations.

NMR characterization of the bioconjugates in DMSO-d_6

The ^{1}H NMR spectrum in DMSO-d_6 of **7**, obtained by either one of the two synthetic approaches, confirmed the excellent purity of the conjugate. A comparison of the spectrum, that presents six well-resolved signals for coordinated cppH in the aromatic region, with those of the two linkage isomers *trans*, *cis*-RuCl$_2$(CO)$_2$(cppH-κN^p) (**1**) and *trans*,*cis*-RuCl$_2$(CO)$_2$(cppH-κN^o) (**2**) strongly suggests that in this bioconjugate the cppH-RRPYIL is bonded through the N^p (Fig. 4, see also Table 1). In fact, all the cppH resonances of **7**, and in particular those of the protons 5 and 6 on the pyrimidine ring that are most affected by differences in the binding mode,[13,14] have chemical shifts very similar to those of **1**. The only exception is the signal of H3′ on the pyridine ring (see below). Accordingly, **7** was formulated as *trans*,*cis*-RuCl$_2$(CO)$_2$(cppH-RRPYIL-κN^p). This finding was rather surprising, considering that in the starting complex **2** cppH was bound through N^o, and implies that, during the SPPS synthesis, isomerization to the N^p species occurred. In addition, the N^p precursor **1** afforded no bioconjugate when reacted with RRPYIL.

The aromatic region of the spectrum of **7** shows also several resonances that have no correlation crosspeaks with carbon resonances in the ^{1}H–^{13}C HSQC spectrum (ESI) and, based on the ^{1}H–^{1}H COSY spectrum, were attributed to NH (or OH) protons of the peptide (see below). The two sharp doublets at *ca.* 7.0 and 6.6 ppm, correlated in the ^{1}H–^{1}H COSY spectrum, belong to the *ortho* and *meta* protons of the tyrosine phenol ring.

The ^{1}H NMR spectra in DMSO-d_6 of [Ru([9]aneS$_3$)(cppH-RRPYIL)(PTA)]$^{2+}$ (**8**) and [Ru([9]aneS$_3$)Cl(cppH-RRPYIL)]$^+$ (**11**) have features similar to that of **7**. In the aromatic region both show a single set of six signals for the metal-bound cppH-RRPYIL and other signals for the amidic protons, partially overlapping with the cppH resonances, that in the ^{1}H–^{1}H COSY spectrum have crosspeaks with protons in the peptide zone (ESI). The comparison of the cppH resonances of **8** and **11** with those of the model complexes with unmodified cppH **4**, **5**, and **10** suggests that in both bioconjugates the cppH-RRPYIL unit is again bound through N^p (see also Table 1). As found for **7**, the resonance of the pyridine ring proton 3′ is downfield shifted compared to the model complexes and falls at higher frequency than 6′ (ESI). The two resonances are easily distinguished in the ^{1}H–^{13}C HSQC spectrum since 3′ has a correlation peak with a carbon atom at *ca.* 127 ppm, while 6′ with a carbon at 155 ppm (see ESI†). Typically, in Ru(II)-cppH complexes the resonances of protons 6 and 6′, that are adjacent to the bonded nitrogen atoms, are the most downfield-shifted signals.[13,14] In this case, some



Scheme 5 Synthesis of bioconjugate **11**.

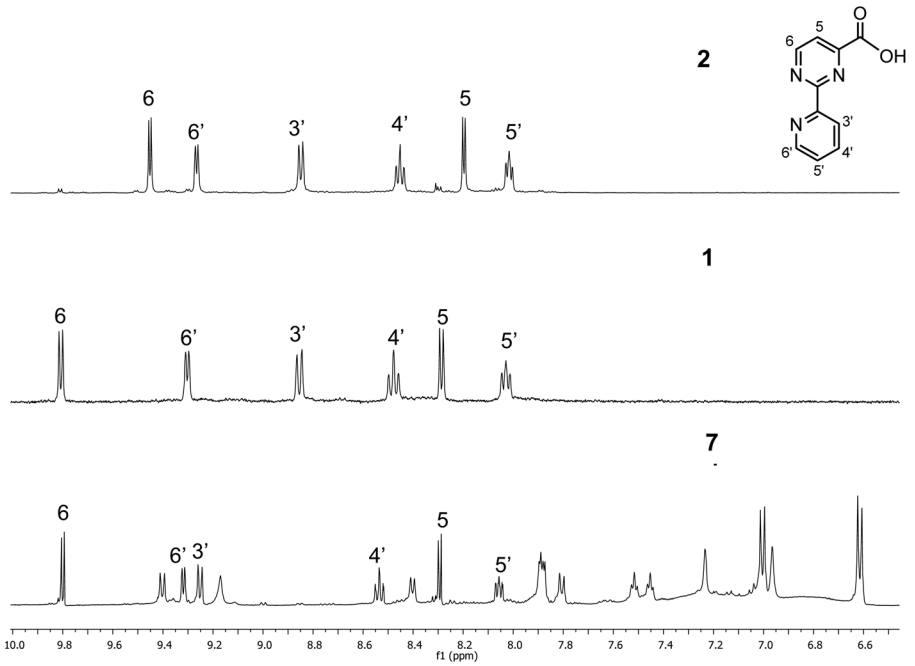


Fig. 4 Aromatic region of the ^{1}H NMR spectra (DMSO-d_6) of *trans,cis*-$RuCl_2(CO)_2$(cppH-κN^o) (**2**) (top), *trans,cis*-$RuCl_2(CO)_2$(cppH-κN^p) (**1**) (middle) and *trans,cis*-$RuCl_2(CO)_2$(cppH-RRPYIL) (**7**) (bottom). Only the resonances of cppH protons are labeled; see insert for labeling scheme.

Table 1 ^{1}H NMR chemical shifts (D_2O) for the resonances of H5 and H6 (pyrimidine ring protons) for model complexes with cppH (both N^p and N^o linkage isomers, when available) and the corresponding bioconjugates with cppH-RRPYIL. $\Delta\delta = \delta_{bioconjugate} - \delta_{complex}$

Compound	H6	H5
trans,cis-$RuCl_2(CO)_2$(cppH-κN^p) (**1**)	9.64	8.22
trans,cis-$RuCl_2(CO)_2$(cppH-RRPYIL) (**7**)	9.79	8.38
$\Delta\delta$	0.15	0.15
trans,cis-$RuCl_2(CO)_2$(cppH-κN^o) (**2**)	9.17	7.72
trans,cis-$RuCl_2(CO)_2$(cppH-RRPYIL) (**7**)	9.79	8.38
$\Delta\delta$	0.62	0.66
$[Ru([9]aneS_3)$(cppH-κN^p)(PTA)$]^{2+}$ (**4**)	9.28	8.16
$[Ru([9]aneS_3)$(cppH-NT)(PTA)$]^{2+}$ (**8**)	9.42	8.26
$\Delta\delta$	0.14	0.10
$[Ru([9]aneS_3)$(cppH-κN^o)(PTA)$]^{2+}$ (**5**)	9.13	7.64
$[Ru([9]aneS_3)$(cppH-NT)(PTA)$]^{2+}$ (**8**)	9.42	8.26
$\Delta\delta$	0.29	0.62
$[Ru([9]aneS_3)Cl$(cppH-κN^p)$]^{+}$ (**10**)	9.52	8.11
$[Ru([9]aneS_3)Cl$(cppH-RRPYIL)$]^{+}$ (**11**)	9.55	8.16
$\Delta\delta$	0.03	0.05

interaction between the peptide and the pyridine ring might be invoked to explain the change of chemical shift for the signal of proton 3′. Finally, the $^{31}P\{^{1}H\}$ NMR spectrum of **8** shows a broad singlet centered at −39.1 ppm, *i.e.* at a frequency consistent with PTA *trans* to S and similar to that of the corresponding model complexes **4** and **5** with unmodified cppH (−34.9 for the N^p isomer and −35.0 for the N^o isomer, recorded in D_2O instead of DMSO-d_6).[14]

In conclusion, according to the above results, the bioconjugate **8** was formulated as $[Ru([9]aneS_3)$(cppH-RRPYIL-κN^p)(PTA)$]^{2+}$ and **11** as $[Ru([9]aneS_3)Cl$(cppH-RRPYIL-κN^p)$]^{+}$.

NMR characterization of the bioconjugates in D_2O

Quite unexpectedly, we found that the ^{1}H NMR spectrum of the bioconjugate **7** presents *twice* the number of cppH-RRPYIL resonances in the aromatic region (Fig. 5) when the compound was dissolved in D_2O rather than in DMSO-d_6. The two sets of four resonances for the protons on the pyridine ring of cppH-NT are well resolved (the assignments of 3′ and 6′ are consistent with the ^{13}C NMR chemical shifts, obtained through the ^{1}H–^{13}C HSQC spectrum), whereas the signals for protons 5 and 6 of the pyrimidine ring are partially or completely overlapping. Each of these integrates as the sum of the two resonances of each proton of the pyridine ring. Thus, the proton spectrum of **7** suggests that in aqueous solution there are two species in *ca.* 3 : 2 ratio (according to the integration of the 3′ and 6′ pairs of resonances) that most likely have the pyrimidine rings in very similar environments (overlapping resonances) and the pyridine rings in relatively different environments. It is worth noting that typically the opposite occurs in case of linkage isomers of cppH, *i.e.* the resonances of the pyrimidine protons are affected much more than those of the pyridine protons by the different binding mode.[13,14] Since in *trans*-$\{RuCl_2\}$ fragments chloride hydrolysis occurs very slowly and to a small extent (and was not observed in the corresponding cppH complexes **1** and **2**), we exclude that the two species derive from this process.

The region of the peptide resonances is obviously more complicated. However, it is possible to distinguish clearly two sets of resonances (two doublets each) for the aromatic protons of Tyr that are basically in the same 3 : 2 intensity ratio as the cppH protons (see ESI†). They are pairwise connected in

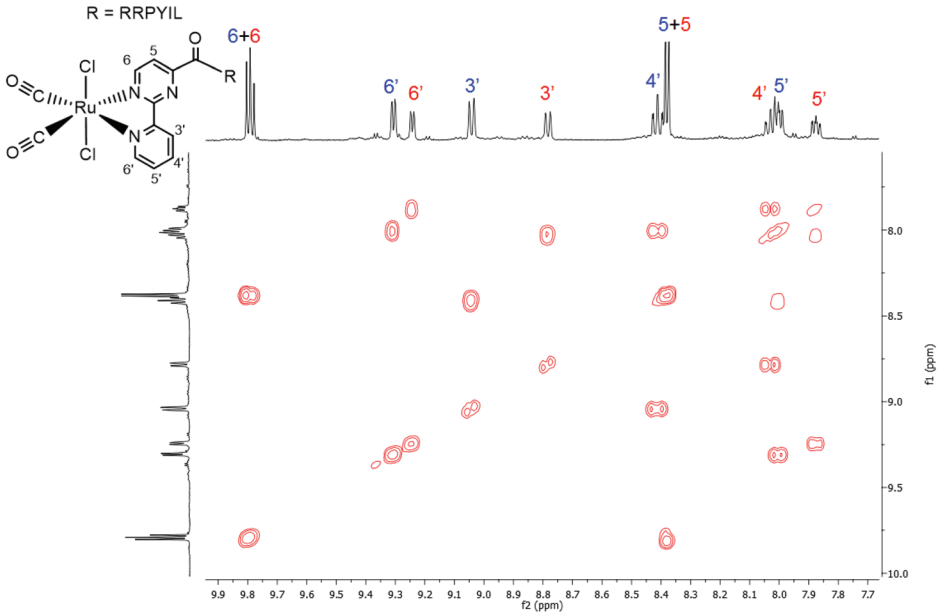


Fig. 5 Aromatic region of the $^{1}H–^{1}H$ COSY spectrum of **7** in D_2O, with labeled cppH resonances (blue for the major isomer, red for the minor one).

the $^{1}H–^{1}H$ COSY spectrum according to their relative intensity. We observe that in D_2O also the signals of the peptide chain in the upfield region are split in two sets, even though not always resolved. The splitting is clearly evident for the CHα and for the leucine and isoleucine methyl resonances (CHδ and CHγ), where differences in chemical shift up to 0.2 ppm are observed between pairs of related signals (ESI). These pairs of related multiplets are also readily spotted in the HSQC spectrum, where they are pairwise coupled to the corresponding carbon signal; in fact, since the ^{13}C spectrum seems less affected than the ^{1}H spectrum, the resonances of the corresponding carbon atoms in the two sets of signals have basically identical chemical shifts.

In order to shed further light on the nature of the two isomers that are observed in aqueous solution, a number of additional NMR experiments were performed. First, an increase in the temperature (up to 65 °C) induced no significant change in the aromatic region of the ^{1}H NMR spectrum, thus suggesting that the two species are not in a conformational equilibrium. Then, the effect of pH on the NMR spectrum was investigated. The pD of the original D_2O solution (*ca.* 3.5) was gradually increased to 8.0 by stepwise addition of small amounts of a NaOD solution, and spectra were recorded after each step. Also in this case no significant change was observed in the aromatic region of the spectrum. Similarly unchanged was the spectrum of the final pD 8.0 solution when it was recorded again at higher temperatures (up to 65 °C).

The ^{1}H NMR spectrum of **8** in D_2O is even more complex than that of **7**. In the aromatic region it is possible to distinguish at least three different sets of resonances, in *ca.* 1 : 1.5 : 2.5 ratio (red/green/blue in Fig. 6), that differ mainly in the shifts of the pyridine ring protons. Two sets (red and green in Fig. 6) are almost completely overlapping, with only the resonances of proton 4′ being resolved. Moreover, each signal of the "blue" set seems to be the sum of two very similar, almost completely overlapping resonances (*e.g.* inspect the signal of H6′ in Fig. 6). In conclusion, compound **8** in aqueous solution is most likely a mixture of four very similar species. Integration of the cppH resonances compared with those of PTA and of the aromatic protons of Tyr, established that in each species PTA is still bound to the ruthenium center and the peptide chain is connected to cppH, *i.e.* each species is integer and has the same coordination environment. Also in this case an increase in temperature (up to 65 °C) and an increase in pD (from *ca.* 3.0 to *ca.* 8.0) left the ^{1}H NMR spectrum virtually unchanged.

The ^{1}H NMR spectrum of **11** in D_2O is further complicated, compared to that of **8**, by the partial hydrolysis of the chloride. In fact, the addition of excess of NaCl (*ca.* 0.5 M) simplifies the spectrum since it reverts the aquation equilibrium, leaving only the resonances of non-hydrolyzed species (Fig. 7). This finding is similar to that observed for the model complex **10** with unmodified cppH (ESI).

From the $^{1}H–^{1}H$ COSY spectrum of the cppH-NT region in **11** it is possible to distinguish at least three different sets of signals for three different major species in *ca.* 1 : 1 : 3 ratio (red/green/blue in Fig. 8). The three species have very similar (overlapping) resonances for the pyrimidine protons 5 and 6 (again suggesting that they are not linkage isomers), whereas several resonances of the pyridine protons are well resolved. In more detail, the two minor species (red and green) have very similar resonances for the pyridine protons, which are rather different from those of the main species (blue). In particular, the resonances of the pyridyl protons 3′ and 4′ of the minor species are shifted upfield compared to those of the main species. Integration of the ^{1}H NMR signals established that in each species the peptide chain is still attached to cppH.

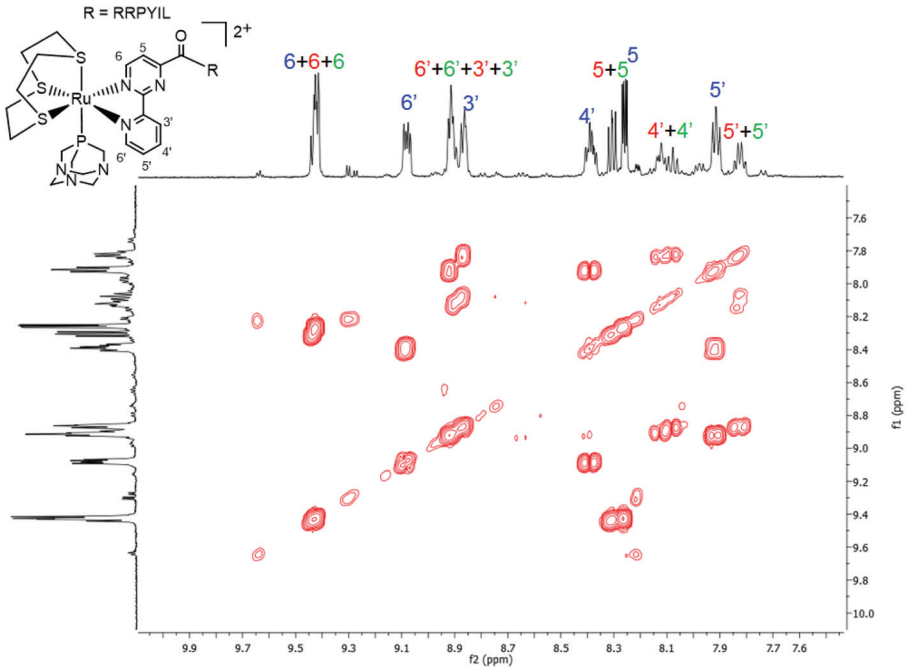


Fig. 6 Downfield region of the $^{1}H–^{1}H$ COSY spectrum in D_2O of [Ru([9]aneS$_3$)(cppH-RRPYIL)(PTA)]$^{2+}$ (**8**) with labeled cppH resonances (a different color for each isomer).

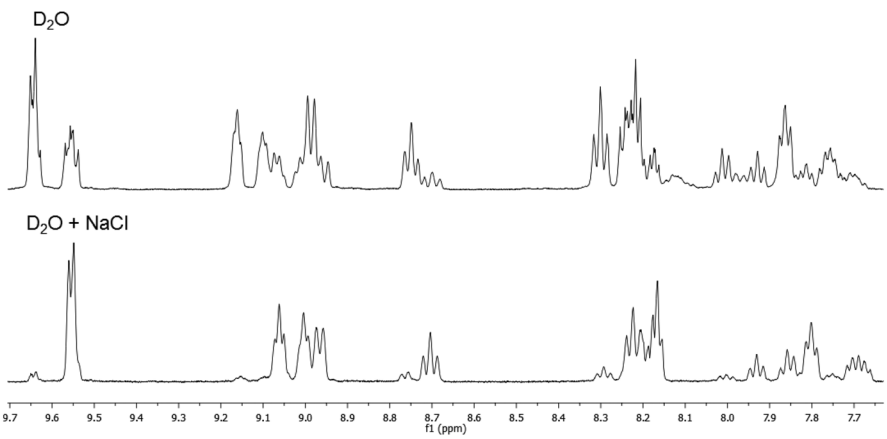


Fig. 7 Downfield region of the ^{1}H NMR spectrum of [Ru([9]aneS$_3$)Cl(cppH-RRPYIL)]$^{+}$ (**11**) after dissolution in D_2O (top) and after addition of an excess of NaCl (bottom).

Finally, in the ^{1}H NMR spectra of **8** and **11** in D_2O the complexity of the cppH region is mirrored by that of the aromatic protons of Tyr (see ESI†). The number of Tyr doublets in the spectra of **8** and **11** is consistent with the presence of three or more species, as indicated by the cppH region.

NMR characterization of cppH-RRPYIL in DMSO-d_6 and D_2O

Intrigued by the results reported above, we performed an NMR characterization of the cppH-RRPYIL ligand alone. Consistent with the above described findings, cppH-RRPYIL shows a single set of signals in DMSO-d_6 (Fig. 9). A similar single set of resonances was found also in CD_3OD. However, in D_2O all resonances – including those of the peptide chain in the upfield region of the spectrum – are split in two sets in *ca.* 1 : 1.7 ratio (Fig. 9), an unambiguous indication that this phenomenon does not depend primarily on the coordination of cppH to ruthenium, even though it seems to be modulated by the coordination environment (different ratio of the isomers for **7**, and additional isomers for **8** and **11**). The spectral patterns of cppH-RRPYIL in each of the two solvents are similar to those observed for **7** (in the corresponding solvent), *i.e.* the conjugate that affords the simplest spectra, thus allowing fruitful comparisons to be made.

The 1D and 2D NMR characterization of cppH-RRPYIL in DMSO-d_6 (ESI), compared with the available literature data,[18] allowed us to locate all the NH signals in the downfield region.

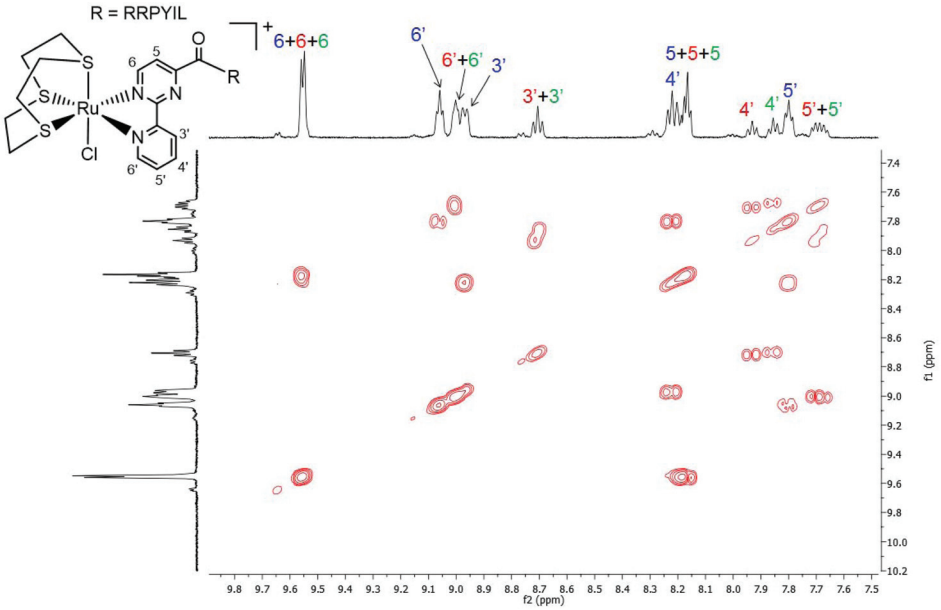


Fig. 8 Downfield region of the $^{1}H–^{1}H$ COSY spectrum in D_2O + NaCl of $[Ru([9]aneS_3)Cl(cppH\text{-}RRPYIL)]^+$ (**11**) with labeled cppH resonances (a different color for each isomer).

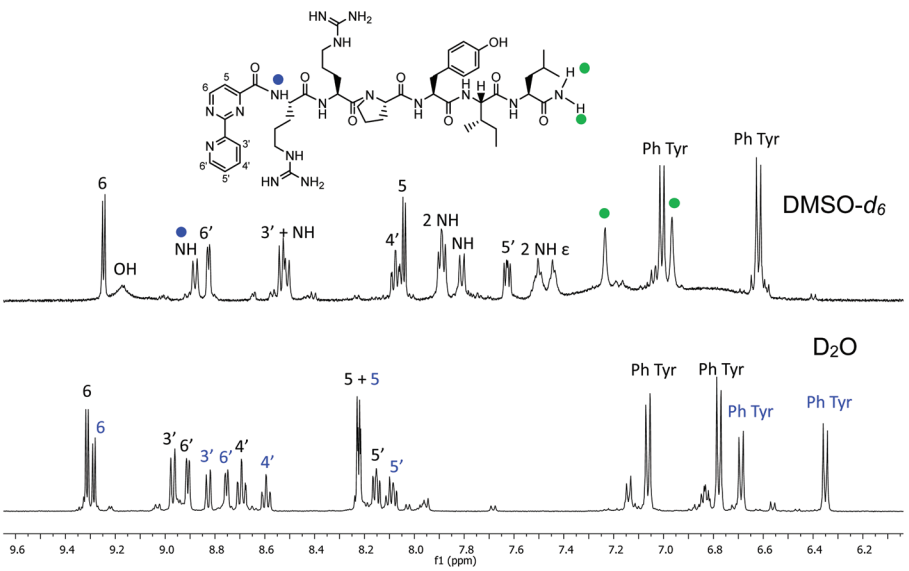


Fig. 9 ^{1}H NMR spectra (aromatic region) of cppH-RRPYIL in DMSO-d_6 (top) and D_2O (bottom). In the latter, the resonances of the major isomer are labeled in black, and those of the minor one in blue.

They have no correlation crosspeaks with carbon resonances in the $^{1}H–^{13}C$ HSQC spectrum (see ESI†). Five of them correlate in the $^{1}H–^{1}H$ COSY spectrum they with the respective Cα protons, whose resonances fall between 4.0 and 4.6 ppm (ESI), and were thus assigned to the amidic protons of the peptide. By comparison with the NMR spectrum of **7** (Fig. 10) we notice that upon coordination to the {*trans*,*cis*-$RuCl_2(CO)_2$} fragment, only the NH resonance at *ca.* 8.8 ppm (blue dot) is downfield shifted (as well as those of cppH, as expected), and it was thus assigned to the amidic proton of the arginine directly bound to cppH. Remarkably, addition of small amounts of D_2O to the DMSO-d_6 solution of cppH-RRPYIL (Fig. 11) induced a large shift of this resonance (blue dot) to higher frequencies (*ca.* 0.8 ppm after addition of 10% D_2O). Taken together, these observations suggest that this proton is involved in an H-bonding interaction with cppH (arguably with the pyridine N atom), that is perturbed by the addition of water. Based on the $^{1}H–^{1}H$ COSY and $^{1}H–^{13}C$ HSQC spectra, and consistent

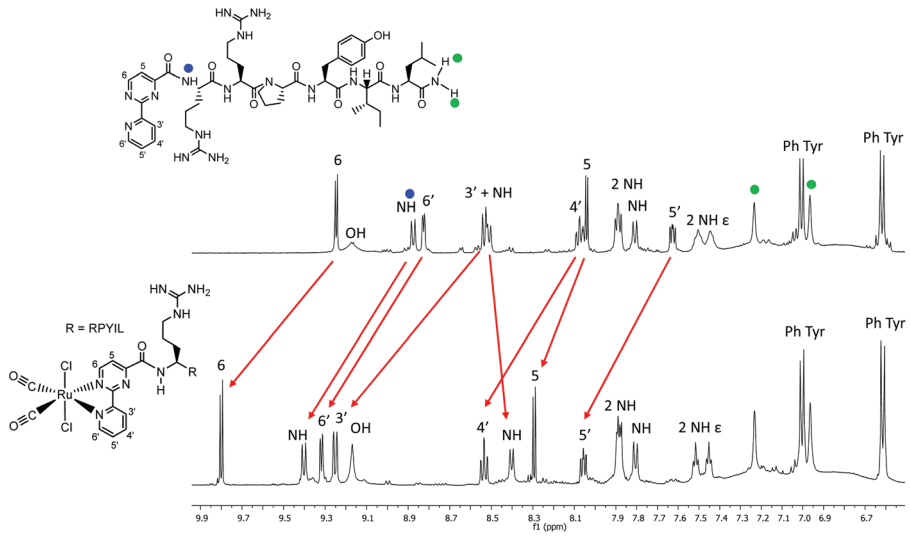


Fig. 10 ^{1}H NMR spectra (aromatic region) of cppH-RRPYIL (top) and *trans,cis*-$RuCl_2(CO)_2$(cppH-RRPYIL) (**7**) (bottom) in DMSO-d_6.

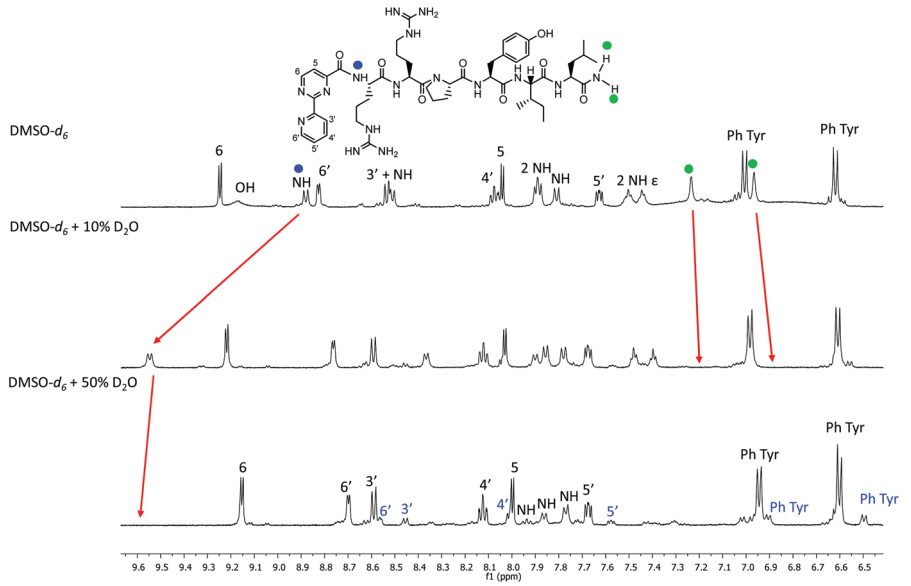


Fig. 11 ^{1}H NMR spectra (aromatic region) of cppH-RRPYIL in DMSO-d_6 (top), with 10% *ca.* of D_2O (middle) and with 50% of D_2O (bottom). The new set of resonances has blue labels.

with literature data,[18] the two multiplets at *ca.* 7.4–7.5 were assigned to the NHε of the two arginines. The rather broad resonance at *ca.* 9.17 ppm, that has no correlation peaks in the ^{1}H–^{1}H COSY and ^{1}H–^{13}C HSQC spectra, was assigned to the proton of the hydroxyl group of the Tyrosine. Furthermore, we found that the two broad singlets at *ca.* 7.2 and 7.0 ppm (green dots) disappear upon addition of very small amounts of D_2O (2%, Fig. 11), and were thus assigned to the terminal $CONH_2$ protons, whose H/D exchange rate is expected to be much faster than that for amidic protons. Further addition of D_2O (up to 50%) led to the progressive disappearance of the NH (and OH) resonances and, more interestingly, to the appearance of a second set of resonances (Fig. 11). Even though in the 1 : 1 DMSO-d_6/D_2O mixture the resonances are hardly comparable to those found in pure D_2O, both in terms of relative intensities and chemical shifts, the trend suggests that the growing "blue" set closely resembles that found in D_2O.

A number of experiments were performed on the pure D_2O solution of cppH-RRPYIL. An increase in temperature up to 65 °C led to a general broadening of all resonances and loss of resolution (ESI), but no clear coalescence between pairs of corresponding resonances in the two sets was observed, suggesting that they do not belong to species in conformational equilibrium. Consistent with this finding, we observed no exchange cross-peaks between the corresponding resonances

in a ROESY spectrum. A three-fold increase in the concentration of cppH-RRPYIL induced no significant change in the ^{1}H NMR spectrum. Finally, a pD titration from *ca.* 3.5 to *ca.* 10.0 led to no significant change in the ratio of the two sets of signals, besides the expected shifts of the cppH resonances due to the deprotonation of the pyridyl nitrogen atom.[14] Furthermore, all changes in chemical shifts were fully reversed upon lowering the pD again.

Overall, the NMR findings with cppH-RRPYIL, that are similar to those observed for the conjugate **7**, are consistent with two hypotheses: (1) either in DMSO-d_6 (and CD_3OD) there is a strong interaction between the pyridine ring of cppH and the peptide chain, leading to a single species, that is partially disrupted by the addition of water, or the opposite is true, *i.e.* (2) an interaction, that is absent in DMSO-d_6 (and CD_3OD), occurs in water. In addition, we believe that such an interaction is intra-rather than inter-molecular (since it does not depend on concentration), and it is not related to hydrogen bonding, since that would be expected to be strongly affected by changes in pH.

In the transition from pure DMSO-d_6 to DMSO-d_6/D_2O mixtures, the downfield region of the spectrum clearly shows that most of the resonances in the growing set are shifted to lower frequencies (resembling what found in pure D_2O). Tyrosine is the amino acid on the peptide sequence whose signals are most affected from the transition from DMSO-d_6 to D_2O, with an upfield shift up to *ca.* 0.4 ppm for both doublets. In the cppH region, the most affected resonances are those of protons 6′ and 3′ on the pyridine ring, with upfield shifts of *ca.* 0.15 ppm from the major ("black") to the minor ("blue") set. These upfield shifts are consistent with the occurrence of a stacking interaction between the phenol ring of tyrosine and the pyridyl ring of cppH. An "educated model" of this intramolecular interaction is presented in Fig. 12.[19] Regretfully, a series of 1D NOE experiments, in which each one of the four Tyr doublets was in turn saturated, afforded no NOE enhancement with the resonances of the pyridine ring protons (as one might expect in case of a stacking interaction).

Numerous attempts to grow single crystals of each conjugate as well as of cppH-RRPYIL by the hanging-drop method, were regretfully also unsuccessful.

Antiproliferative and antimicrobial activity

The antiproliferative properties of conjugates **7**, **8**, and **11** (as well as of the Ru-cppH model compounds **1–4** for comparison) were assessed against three different cell lines:[20] colon adenocarcinoma (HT-29), breast cancer (MCF-7) and pancreatic cancer (PT-45). All the cell lines were treated for 48 hours with increasing concentrations of each compound. None of the investigated compounds showed antiproliferative activity, even at the highest investigated concentration of 200 μM. Given the absence of activity, the targeting properties of the conjugates were not investigated.

The antimicrobial activity was assessed on a representative selection of Gram-positive (*B. subtilis* and two strains of *S. aureus*) and Gram-negative (*E. coli*, *A. baumannii* and *P. aeruginosa*) bacterial strains. The lowest concentration inhibiting visible growth was reported as minimal inhibitory concentration (MIC, see ESI†). Compounds **1–4** were found to be inactive against both Gram-positive and Gram-negative strains. Among the bioconjugates, **11** resulted to be the most active, with a moderate activity against *E. coli* (MIC = 32 μg mL^{-1}) and a low activity against *B. subtilis* (MIC = 256 μg mL^{-1}).[21]

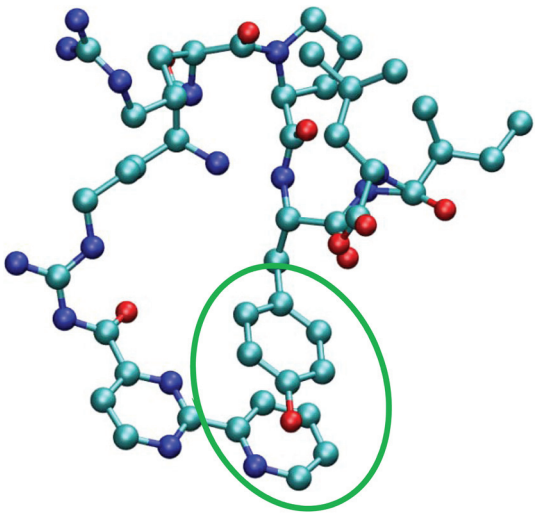


Fig. 12 Model (H atoms omitted) of the possible intramolecular stacking interaction occurring in aqueous solution between the phenol ring of tyrosine and the pyridyl ring of cppH in cppH-RRPYIL. For the sake of clarity this cartoon, even though realistic,[19] is just a suggestion, and not supported by *e.g.* NOE or ROESY NMR data.

Conclusions

The diimine cppH linker was exploited for preparing three new Ru(II) bioconjugates with the C-terminal hexapeptide sequence of neurotensin, RRPYIL, namely *trans, cis*-$RuCl_2(CO)_2$(cppH-RRPYIL-κN^p) (**7**), [Ru([9]aneS_3)(cppH-RRPYIL-κN^p)(PTA)][$Cl)_2$ (**8**), and [Ru([9]aneS_3)Cl(cppH-RRPYIL-κN^p)]Cl (**11**). Two complementary synthetic approaches were investigated: (i) attachment of RRPYIL to the carboxylic group of cppH on pure Ru-cppH linkage isomers (**7**), and (ii) preparation of cppH-RRPYIL followed by its coordination to suitable Ru(II) precursors with two adjacent labile ligands (**7**, **8**, **11**).

Since cppH is capable of forming stereoisomers, depending on whether the nitrogen atom *ortho* (N^o) or *para* (N^p) to the carboxylate on C4 in the pyrimidine ring is used for metal binding, one of the aims of this work was that of obtaining pairs of stereoisomeric conjugates (*i.e.* linkage isomers) and evaluating their biological activity. A thorough NMR characterization clearly indicated that in all cases, regardless of the synthetic procedure adopted and of the stereochemistry of the precursor, exclusively N^p linkage-isomers were obtained in pure form, even when N^o isomers were used as starting material. However, the NMR studies provided also unexpected

findings: whereas in DMSO-d_6 the NMR spectra are rather straightforward and present a single set of resonances, the spectra of the same samples in D_2O show that, in this solvent, each bioconjugate exists as two (**7**) or even three if not four (**8** and **11**) very similar species (each one corresponding to the integer compound). The spectra were not affected by an increase in temperature and by changing the pH of the solution. Since the NMR spectra of the different species differ mainly in the resonances of the pyridine ring of cppH-RRPYIL, we argue that they do not differ in the Ru coordination environment (including cppH linkage isomers) but derive likely from some strong intra- or intermolecular interaction between the peptide and the pyridine ring of cppH (see below).

This conclusion was confirmed by the NMR investigation performed on the cppH-RRPYIL ligand alone: it exists as a single species in DMSO-d_6 (and CD_3OD), but as two species in *ca.* 1 : 1.7 ratio in D_2O. Overall, the NMR findings with cppH-RRPYIL, that are similar to those observed for **7**, are consistent with the occurrence of a strong intramolecular stacking interaction between the phenol ring of tyrosine and the pyridyl ring of cppH (Fig. 12). Stacking interactions between aromatic rings are expected to be stronger in water (solvophobic effect). This interaction, that leads to two stereoisomeric species in the free cppH-RRPYIL ligand and in the bioconjugate **7**, is somehow modulated by the less symmetrical Ru coordination environments in **8** and **11**, affording three-four very similar species.

Firm proof for our interpretation could be obtained from a single crystal X-ray structure. However, even though we tried hard, no suitable crystals could be obtained. So, from the NMR data alone, we can deduce the above conclusions, but these – even though fully consistent with all experimental data – certainly have a greater degree of uncertainty than an X-ray structure would have, and we are unable to establish the stereochemical differences between the stereoisomers found in D_2O. Nevertheless, we stress that detailed NMR investigations of metal bioconjugates are virtually absent in the literature, thus impeding comparisons: the nature and purity of the conjugates are typically assessed only by mass spectroscopy and HPLC, respectively. We also remark that the phenomenon of stereoisomer formation occurs exclusively in D_2O, and that it would have escaped detection by an NMR investigation performed in DMSO-d_6 only. Therefore, our findings strongly suggest that in the future the characterization of new metal conjugates – as well as the reinvestigation of known ones – should include detailed NMR investigations in multiple solvents, including D_2O. It would be of particular interest to establish if the formation of stereoisomers in aqueous solutions is a characteristic feature of cppH-RRPYIL or if it occurs also with other linker-peptide combinations.

Finally, neither the bioconjugates **7**, **8**, and **11** nor the Ru-cppH model compounds **1–4** showed any significant antiproliferative or antibacterial activity. For comparison, NT_{8-13} metal bioconjugates with the $Co_2(CO)_8$ fragment or a Pt(IV) complex were previously shown to be moderately toxic towards some cancer cell lines.[5,8]

Experimental section

Materials

All chemicals were purchased from Sigma-Aldrich and used as received. Solvents were of reagent grade. The precursors *trans, cis,cis*-$RuCl_2(CO)_2$(dmso-O)$_2$ (**3**),[22] Ru([9]aneS_3)Cl_2(PTA) (**6**),[23] and Ru([9]aneS_3)Cl_2(dmso-S) (**9**),[24] and the linker cppH·HNO_3,[10] were synthesized as described in the literature.

Instrumental methods

Mono- and bi-dimensional (^{1}H–^{1}H COSY, ^{1}H–^{13}C HSQC) NMR spectra were recorded at room temperature on a Varian 400 or 500 spectrometer (^{1}H: 400 or 500 MHz, ^{31}P{^{1}H}: 161 or 202 MHz). ^{1}H chemical shifts were referenced to the peak of residual non-deuterated solvent (δ = 2.50 for DMSO-d_6 and 3.31 for CD_3OD) or were measured relative to the internal standard DSS (δ = 0.00) for D_2O. Carbon resonances were assigned through the HSQC spectra. ^{31}P{^{1}H} chemical shifts were referenced to external 85% H_3PO_4 at 0.00 ppm. ESI mass spectra were collected in the positive mode on a Bruker Esquire 6000 spectrometer. Elemental analysis for **10** was performed on a Thermo Flash 2000 CHNS/O analyzer in the Department of Chemistry of the University of Bologna (Italy).

Analytical HPLC was performed on a Smartline instrument (Knauer) with a Reprosil-Pur C-18 reversed-phase column at a flow rate of 1.0 mL min^{-1}. Preparative HPLC was performed on a Varian Prostar with a HIBAR Lichrospher 100 RP-18e reversed-phase column at a flow rate of 20 mL min^{-1}. Analytical and preparative runs were performed with a linear gradient of 5% buffer B per min starting at 5 min of buffer A (buffer A = H_2O/MeCN/TFA 95 : 5 : 0.1, v/v/v, buffer B = MeCN/H_2O/TFA 95 : 5 : 0.1, v/v/v) was used.

Lyophilization was performed on an ALPHA 1–4 LDplus lyophilizator (Martin Christ, Osterode am Harz, Germany). Attempts to grow crystals of each conjugate as well as of cppH-RRPYIL by the hanging-drop method were done at different pH values (pH = 5 and 5.5 (sodium citrate), pH = 6 and 6.5 (MES = 2-(*N*-morpholino)ethanesulfonic acid), pH = 8 (TRIS = tris(hydroxymethyl)aminomethane)), in the presence of different salts (NaCl (from 0.1 to 0.3 M), $MgCl_2$ (from 0.1 to 0.5 M), and $(NH_4)_2SO_4$ (from 10% to 60% of a saturated solution)), and using two types of PEG (PEG300, and PEG4000) as dehydrating agent.

Cell cultures and cytotoxicity

Dulbecco's Modified Eagle's Medium (DMEM), containing 10% fetal calf serum, and 1% penicillin and streptomycin, was used as growth medium. MCF-7, HT-29 and PT-45 cells were detached from the wells with trypsin and EDTA, harvested by centrifugation and re-suspended again in cell culture medium. The assays have been carried out on 96 well plates with 6000 cells per well for each cell line. After 24 h of incubation at 37 °C and 10% CO_2, the cells were treated with the compounds **1–4**, **7**, **8**, and **11** at 1, 10, 20, 40, 80, 100, 200 μM concentration (with DMSO concentrations of 0.5%) with a final volume of 200 μL per well. For a negative control, one series of cells was

left untreated. The cells were incubated for 48 h followed by adding 50 μL MTT (2.5 mg mL^{-1}). After an incubation time of 2 h, the medium was removed and 200 μL DMSO were added. The formazan crystals were dissolved and the absorption was measured at 550 nm, using a reference wavelength of 620 nm.

Antimicrobial activity

The antimicrobial activity of compounds **1–4**, **7**, **8**, **11** was evaluated by determining of the minimal inhibitory concentration (MIC, μg mL^{-1}) according to a previously described method.[25] *Bacillus subtilis* DSM 402, *Staphylococcus aureus* DSM 20231, and *Staphylococcus aureus* ATCC 43300 were chosen as Gram-positive test strains. *Escherichia coli* DSM 30083, *Acinetobacter baumannii* DSM 30007, and *Pseudomonas aeruginosa* DSM 50071 served as Gram-negative strains in these experiments. With the exception of *P. aeruginosa* (Mueller-Hinton II broth), all strains were cultured in Mueller-Hinton broth. The compounds were dissolved in DMSO to obtain stock solutions of 10 mg mL^{-1}. Serial dilution was carried out with a Tecan Freedom Evo 75 liquid handling workstation (final concentrations range: 0.5 to 512 μg mL^{-1}). The obtained serial dilutions were inoculated with 5×10^5 bacteria per mL taken from late exponential cultures grown in the above mentioned media (total volume 200 μL per well). Cells were incubated for 16–18 h at 37 °C. The lowest concentration inhibiting visible bacterial growth is given as MIC.

X-ray diffraction

Data collections were performed at the X-ray diffraction beamline (XRD1) of the Elettra Synchrotron of Trieste (Italy) equipped with a Pilatus 2 M image plate detector.

Collection temperature was 100 K (nitrogen stream supplied through an Oxford Cryostream 700); the wavelength of the monochromatic X-ray beam was 0.700 Å and the diffractograms were obtained with the rotating crystal method. The crystals were dipped in N-paratone and mounted on the goniometer head with a nylon loop. The diffraction data were indexed, integrated and scaled using the XDS code.[26] The structures were solved by the dual space algorithm implemented in the SHELXT code.[27] Fourier analysis and refinement were performed by the full-matrix least-squares methods based on F^2 implemented in SHELXL.[28] The Coot and SHELXLE programs were used for modeling.[29,30] Anisotropic thermal motion was allowed for all non-hydrogen atoms. Hydrogen atoms were placed at calculated positions with isotropic factors $U = 1.2 \times U_{eq}$, U_{eq} being the equivalent isotropic thermal factor of the bonded non hydrogen atom. Crystal data and details of refinements are in the ESI.†

Solid-phase synthesis of the hexapeptide RRPYIL

The hexapeptide RRPYIL was synthesized manually at room temperature by using a Fmoc-protecting strategy and a Rink amide resin. Reactions were carried out in polypropylene syringes on a mechanical shaker. The resin was swollen in DMF before use.

Fmoc-deprotection: 20% piperidine in DMF (5 mL) was added to the resin for 2 × 10 min. After each coupling and deprotection step, the resin was thoroughly washed with DMF, DCM and DMF (5 mL each).

Coupling: Fmoc-protected amino acids (4 equiv.: 648.7 mg for Arg, 337.4 mg for Pro, 459.5 mg for Tyr, 353.4 mg for Ile and Leu) were pre-activated with TBTU (234.1 mg, 4 equiv.), HOBT (135.1 mg, 4 equiv.) and DIPEA (349 μL, 8 equiv.) in DMF (1–2 mL) for 1 min, mixed with the resin and shaken for 1 h.

Final cleavage from the resin and side chain deprotection of the peptide was accomplished by using a mixture of 90% TFA, 5% TIS and 5% H_2O (3 mL) for 4 h at room temperature. The cleaved peptide was precipitated with ice-cold diethyl ether (30 mL), centrifuged and washed two times with diethyl ether (30 mL), and then lyophilized and purified by preparative HPLC. [$C_{38}H_{65}N_{13}O_7$]: (M_W: 815.51). Analytical HPLC: t_R = 14.8 min, ESI mass spectrum: 816.6 *m*/*z*, $[M + H]^+$ (calcd 816.10).

Synthesis of cppH-RRPYIL

The coupling of the hexapeptide RRPYIL with cppH·HNO_3 was performed as with the aminoacids: cppH (211.3 mg, 4 equiv.) was pre-activated with TBTU (187.2 mg, 4 equiv.), HOBT (108.1 mg, 4 equiv.) and DIPEA (175 μL, 5 equiv.) in DMF (1–2 mL) for 1 min, mixed with the resin and shaken for 2 h. Final cleavage from the resin and side chain deprotection of the peptide was accomplished by using a mixture of 90% TFA, 5% TIS and 5% H_2O (2.8 mL) for 4 h at room temperature. The cleaved cppH-RRPYIL was precipitated with ice-cold diethyl ether (30 mL), centrifuged and washed two times with diethyl ether (30 mL), and then lyophilized and purified by preparative HPLC. Yield of purified product: 120.8 mg (0.120 mmol, 62%). [$C_{48}H_{70}N_{16}O_8$]: (M_W: 998.54). Selected ^{1}H NMR resonances (DMSO-d_6), δ (ppm): 9.25 (d, 1H, H6), 9.17 (br s, 1H, OH Tyr), 8.88 (d, 1H, NH), 8.82 (d, 1H, 6′), 8.52 (m, 2H, 3′ + NH), 8.06 (m, 2H, 4′ + 5), 7.89 (m, 2H, NH + NH), 7.81 (d, 1H, NH), 7.63 (t, 1H, 5′), 7.50 (m, 1H, NHε Arg), 7.44 (m, 1H NHε Arg), 7.01, 6.62 (d, 4H, H2,6 and H5,3 Tyr), 4.7–4.1 (m, 6H, Hα peptide). Selected ^{13}C NMR resonances from the HSQC spectrum (DMSO-d_6), δ (ppm): 161.0 (C6), 150.2 (C6′), 138.0 (C4′), 130.5 (C Tyr), 126.1 (C5′), 124.4 (C3′), 118.0 (C5), 115.6 (C Tyr), 59.6–50.6 (Cα peptide). Analytical HPLC: t_R = 16.4 min, ESI mass spectrum: 999.7 *m*/*z*, $[M + H]^+$ (calcd 999.0).

Synthesis of the compounds

***trans*,*cis*-$RuCl_2(CO)_2$(cppH-RRPYIL-κN^p) (7).** This bioconjugate was prepared using two different approaches: (1) by SPPS between *trans*,*cis*-$RuCl_2(CO)_2$(cppH-κN^o) (**2**) and RRPYIL, and (2) by treating the *trans*,*cis*,*cis*-$RuCl_2(CO)_2$(dmso-O)$_2$ (**3**) precursor with an equimolar amount of cppH-RRPYIL in aqueous solution.

(1) The SPPS coupling between the hexapeptide RRPYIL and *trans*,*cis*-$RuCl_2(CO)_2$(cppH-κN^o) (**2**) was performed using PyBOP/DIPEA/Ru(II): compound **2** (68.7 mg, 5 equiv.) was pre-activated with PyBOP (83.3 mg, 5 equiv.) and DIPEA (55 μL, 10

equiv.) in DMF (1 mL) for 1 min, mixed with the resin and shaken for two days in light protected conditions. The cleavage and deprotection were done using TFA/phenol/TIS in 85 : 10 : 5 ratio (450 μL) for 2 h. The cleaved compound was precipitated with ice-cold diethyl ether (5 mL), centrifuged and washed two times with diethyl ether (5 mL) and then lyophilized and purified using semi-preparative HPLC. Yield of purified product: 10.9 mg (0.02 mmol, 62%).

(2) A 15.0 mg amount of *trans*,*cis*,*cis*-$RuCl_2(CO)_2$(dmso-O)$_2$ (**3**) (0.039 mmol) was partially dissolved in 2.5 mL of water. One equivalent of cppH-RRPYIL (39.0 mg, 0.039 mmol) was added and the mixture was stirred for two days at room temperature in light protected conditions. A clear yellow solution was obtained in 1 h, and then the color turned progressively to orange. The final solution was lyophilized and purified using semi-preparative HPLC. Yield of purified product: 28.8 mg (0.023 mmol, 60%). [$C_{50}H_{70}N_{16}Cl_2O_{10}Ru$]: ($M_W$: 1226.6). Selected ^{1}H NMR resonances (DMSO-d_6), δ (ppm): 9.81 (d, 1H, H6), 9.40 (d, 1H, NH), 9.31 (d, 1H, H6′), 9.15 (d, 1H, H3′), 9.17 (br s, 1H, OH Tyr), 8.53 (t, 1H, H4′), 8.40 (d, 1H, NH), 8.29 (d, 1H, H5), 8.06 (t, 1H, H5′), 7.88–7.82 (m, 4H, NH), 6.99, 6.62 (d, 4H, H2,6 and 5,3 Tyr) 4.65–4.10 (m, 6H, Hα peptide). Selected ^{13}C NMR resonances from the HSQC spectrum (DMSO-d_6), δ (ppm): 163.3 (C6), 154.2 (C6′), 141.4 (C4′), 130.9 (C5′), 130.2 (C Tyr), 127.6 (C3′), 121.0 (C5), 115.5 (C Tyr), 59.5–50.6 (Cα peptide). Analytical HPLC: t_R = 18.2 min, ESI mass spectrum: 1227.3 m/z, $[M + H]^+$ (calcd 1227.0).

[Ru([9]aneS$_3$)(cppH-RRPYIL-κ*N*^{p})(PTA)]Cl$_2$ (8). A 25.0 mg amount of Ru([9]aneS$_3$)Cl$_2$(PTA) (**6**, 0.050 mmol) was dissolved in 1 mL of water. One equivalent of cppH-NT (50.0 mg, 0.050 mmol) was added and the mixture was heated at 80 °C for 8 h. The clear yellow solution, obtained within minutes, turned progressively orange. The final solution was lyophilized and purified using semi-preparative HPLC. Yield of purified product: 43.2 mg (0.03 mmol, 60%). [$C_{60}H_{94}N_{19}Cl_2O_8PS_3Ru$]: ($M_w$: 1509.5). Selected ^{1}H NMR resonances (DMSO-d_6), δ (ppm): 9.27 (m, 4H, H6 + H3′ + NH + OH Tyr), 8.82 (d, 1H, H6′), 8.43 (m, 2H, H4′ + NH), 8.12 (d, 1H, H5), 7.89 (m, 2H, H5′ + NH), 7.83 and 7.74 (2d, 2H, NH), 7.01 and 6.62 (2d, 4H, H2,6 and 5,3 Tyr) 4.70–4.10 (m, 6H, Hα peptide). Selected ^{13}C NMR resonances from the HSQC spectrum (DMSO-d_6), δ (ppm): 163.4 (C6), 154.8 (C6′), 140.0 (C4′), 130.9 (C5′), 130.5 (Tyr), 127. 9 (C3′), 121.4 (C5), 115.2 (Tyr), 59.5–51.1 (Cα peptide). ^{31}P{^{1}H} NMR spectrum (DMSO-d_6), δ (ppm): −39.1 (br s, 1P, PTA *trans* to S). Analytical HPLC: t_R = 16.6 min, ESI mass spectrum: 1438.6 m/z, $[M - H^+]^+$ (calcd 1438.0).

[Ru([9]aneS$_3$)Cl(cppH-κ*N*^{p})]Cl (10). A 30.0 mg amount of Ru([9]aneS$_3$)Cl$_2$(dmso-S) (**9**) (0.068 mmol) was dissolved in 2 mL of water. One equivalent of cppH (18.6 mg, 0.068 mmol) was added and the mixture was heated to reflux. The clear orange solution obtained within minutes turned rapidly red. After 5 h the solution was concentrated to *ca.* 1 mL. X-ray quality crystals of the product formed within two days at room temperature. They were removed by filtration, washed with cold water and acetone and dried *in vacuo* (yield: 30.1 mg, 80%). Elemental analysis calcd for [$C_{16}H_{19}N_3Cl_2O_2S_3Ru$] ($M_W$: 552.9): C 34.72; H 3.46; N 7.59. Found: C 34.76; H 3.40; N 7.52. ^{1}H NMR (D_2O + NaCl), δ (ppm): 9.47 (d, 1H, H6), 9.04 (d, 1H, H6′), 8.89 (d, 1H, H3′), 8.22 (t, 1H, H4′), 8.13 (d, 1H, H5), 7.78 (t, 1H, H5′), 2.85 (m, 12H, [9]aneS$_3$). Selected ^{13}C NMR resonances from the HSQC spectrum (D_2O + NaCl), δ (ppm): 162.0 (C6), 152.9 (C6′), 138.8 (C4′), 129.0 (C5′), 126.4 (C3′), 121.4 (C5), 31.2 ([9]aneS$_3$). ESI mass spectrum: 516.2 m/z, $[M]^+$ (calcd 516.0).

[Ru([9]aneS$_3$)Cl(cppH-RRPYIL-κ*N*^{p})]Cl (11). A 15.0 mg amount of Ru([9]aneS$_3$)Cl$_2$(dmso-S) (**9**) (0.034 mmol) was dissolved in 1 mL of water. One equivalent of cppH-NT (17.0 mg, 0.034 mmol) was added and the mixture was heated at 80 °C for 5 h. The clear yellow solution, obtained within minutes, turned rapidly red. The final solution was lyophilized and purified using semi-preparative HPLC. Yield of purified product: 30.8 mg (0.02 mmol, 65%). [$C_{54}H_{82}N_{16}Cl_2O_8S_3Ru$]: ($M_w$: 1350.9). Selected ^{1}H NMR resonances (DMSO-d_6), δ (ppm): 9.51 (d, 1H, H6), 9.29 (m, 1H, NH), 9.18 (br s, 1H, OH Tyr), 9.11 (d, 1H, H3′), 9.04 (d, 1H, H6′), 8.65 (d, 1H, NH), 8.38 (m, 1H, NH), 8.31 (t, 1H, H4′), 8.11 (d, 1H, H5), 7.89 (m, 1H, NH), 7.82 (m, 2H, H5′ + NH), 6.99 and 6.60 (2d, 4H, H2,6 and 5,3 Tyr), 4.70–4.10 (m, 6H, Hα peptide). Selected ^{13}C NMR resonances from the HSQC spectrum (DMSO-d_6), δ (ppm): 163.2 (C6), 153.8 (C6′), 138.6 (C4′), 130.5 (C Tyr), 129.6 (C5′), 126.9 (C3′), 120.0 (C5), 115.2 (C Tyr), 59.5–50.7 (Cα peptide). Analytical HPLC: t_R = 17.5 min, ESI mass spectrum: 1315.8 m/z, $[M]^+$ (calcd 1315.1).

Conflicts of interest

There are no conflicts to declare.

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