JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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The Chemoselective Reactions of Tyrosine-Containing G-Protein-Coupled Receptor Peptides with $[Cp^*Rh(H_2O)_3](OTf)_2$, Including 2D NMR Structures and the Biological Consequences

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Supporting Information

ABSTRACT: The bioconjugation of organometallic complexes with peptides has proven to be a novel approach for drug discovery. We report the facile and chemoselective reaction of tyrosine-containing G-protein-coupled receptor (GPCR) peptides with $[Cp^*Rh(H_2O)_3](OTf)_2$, in water, at room temperature, and at pH 5−6. We have focused on three important GPCR peptides; namely, $[Tyr^1]$-leu-enkephalin, $[Tyr^4]$-neurotensin(8-13), and $[Tyr^3]$-octreotide, each of which has a different position for the tyrosine residue, together with competing functionalities. Importantly, all other functional groups present, i.e., amino, carboxyl, disulfide, phenyl, and indole, were not prominent sites of reactivity by the Cp*Rh tris aqua complex. Furthermore, the influence of the Cp*Rh moiety on the structure of $[Tyr^3]$-octreotide was characterized by 2D NMR, resulting in the first representative structure of an organometallic-peptide complex. The biological consequences of these Cp*Rh-peptide complexes, with respect to GPCR binding and growth inhibition of MCF7 and HT29 cancer cells, will be presented for $[(\eta^6\text{-Cp}^*\text{Rh-Tyr}^1)\text{-leu-enkephalin}](OTf)_2$ and $[(\eta^6\text{-Cp}^*\text{Rh-Tyr}^3)\text{-octreotide}](OTf)_2$.

The bioorganometallic chemistry discipline has clearly demonstrated that organometallic chemistry is compatible, at the interface, with biology.[1] One current topic, the bioconjugation of important peptides to organometallic complexes, has been studied extensively.[2] In general, the organometallic complexes have been bioconjugated terminally, to either the free amino or carboxyl groups of the designated peptide, which could potentially have adverse affects on their bioactivity, while the majority of these bioconjugation reactions were conducted in organic solvents.[2] Several recent studies have shown that water, a biologically compatible solvent, was found to be a medium for the bioconjugation of aromatic amino acids and several peptides with organometallic complexes.[3]

For example, Grotjahn et al. have reacted a 27 amino acid peptide, secretin, with a $['Cp\{(CH_2)_2NH_2\}Ru(CH_3CN)_2]^+$ derivative to provide, at room temperature, the η^6 product with the only aromatic residue, phenylalanine, via competition with the 'Cp side-chain-NH_2; an apparently air sensitive synthetic method.[3a,b] Importantly, these studies also did not address the chemoselectivity for other aromatic amino acid-containing peptides, via intramolecular competition experiments.[3a,b]

More recently, $[\eta^6\text{-CpRu(naphthalene}]^+$ was shown to react under visible-light irradiation, via an η^6 ligand exchange reaction, with aromatic amino acids and several peptide substrates, angiotensin I and II, in water.[3c] However, from our perspective, the stringent parameters reported for obtaining reaction products, by using photochemical techniques, with lengthy irradiation periods (8−36 h), have significant limitations for general synthetic applications, while also not demonstrating chemoselectivity for a specific aromatic amino acid in peptides; these authors had shown that in a competition with individual aromatic amino acids, phenylalanine, tyrosine, and tryptophan, the $[\eta^6\text{-CpRu-tryptophan}]^+$ complex dominated by a combined ratio of 4:1, over the other two competitors.[3c]

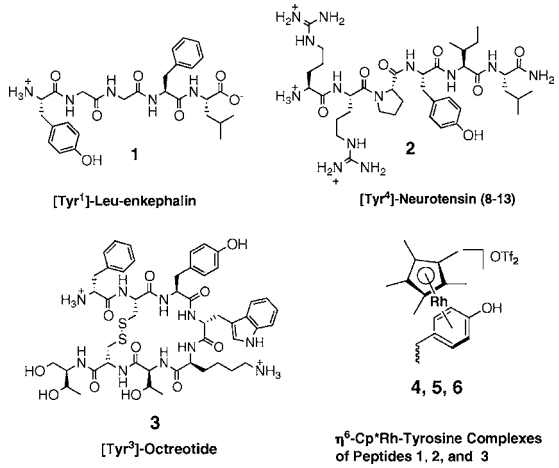
The phenol side chain of tyrosine-containing peptides has been shown to be a potential component for molecular recognition, as well as biological activity, with certain G-protein-coupled receptors (GPCRs).[4] Moreover, GPCRs have been shown to influence the physiological responses to hormones, neurotransmitters, and environmental stimulants; and therefore, have importance as therapeutic targets for a wide spectrum of diseases.[4] For example, tyrosine kinases[5] have been involved in autoimmune diseases[6a] and cancer,[6b] while tyrosine residues, in proteins, were shown to be crucial in some electron-transfer pathways, or at the active sites of some enzymes.[6c]

Received: March 30, 2012
Published: June 7, 2012

dx.doi.org/10.1021/ja303010k | J. Am. Chem. Soc. 2012, 134, 10321−10324

Surprisingly, the tyrosine residue of peptides had not been previously considered as a site of reactivity with organometallic reagents, in competition with other aromatic amino acid residues. Therefore, we wish to report a facile, chemoselective bioconjugation of important tyrosine amino acid components of GPCRs peptides, in water, at room temperature, as a function of pH, by utilizing an air and water stable organometallic aqua complex, $[Cp^*Rh(H_2O)_3](OTf)_2$.[7] The important tyrosine-containing GPCR peptides that were used to demonstrate this chemoselective bioconjugation technique are shown in the Chart 1, and they include, [Tyr^1]-leu-enkephalin, **1**, [Tyr^4]-neurotensin(8-13), **2**, and [Tyr^3]-octreotide, **3**, with the tyrosine residue being a potential molecular recognition component for binding to their respective receptor sites, but found not to be always crucial.[4b,6] We were also interested in being able to ascertain the biological consequences of these synthetic transformations, by conducting various bioassays of these Cp*Rh-tyrosine peptide complexes with GPCR cells, as well as hormone dependent cancer cells.

Chart 1. Tyrosine-Containing Peptides, 1−3, and Their Corresponding η^6-Cp*Rh-Tyrosine Complexes, 4−6



The first example of this new bioconjugation technique was the reaction of a 2-fold excess of $[Cp^*Rh(H_2O)_3](OTf)_2$ with the opioid receptor neuropeptide, [Tyr^1]-leu-enkephalin, **1** (Chart 1), which showed the high chemoselectivity to tyrosine, in the presence of a phenylalanine component, by ^{1}H and ^{13}C NMR. Thus, the ^{1}H and ^{13}C NMR spectra, pD = 5.5 (pD = pH + 0.4, D_2O), demonstrated the characteristic upfield shifts for the η^6-Cp*Rh-phenol protons/carbon atoms, in comparison to the tyrosine residue of peptide **1**.[8] Thus, all starting [Tyr^1]-leu-enkephalin, **1**, was converted to $[(\eta^6\text{-Cp*Rh-Tyr}^1)\text{-leu-enkephalin}](OTf)_2$, **4** (dicationic charge only pertains to the η^6-Cp*Rh-phenol complex in **4**−**6**; Chart 1), with no indication of phenylalanine reactivity; the HPLC isolated yield was 65% from a larger scale reaction at pH 5−5.5 (see the Supporting Information (SI) for the synthesis and ^{1}H and ^{13}C NMR, 2D TOCSY and ROESY NMR, and HR-ESI-MS data).

We then extended this approach to [Tyr^4]-neurotensin(8-13), **2**, which has been shown to regulate hypotension, hyperglycemia, and hypothermia.[9] Therefore, the reaction of **2**, with the Cp*Rh tris aqua complex provided an ^{1}H NMR spectrum, as well as HR-ESI-MS data, complex **5** (Chart 1), which were consistent with tyrosine residue chemoselectivity; ^{1}H NMR studies in D_2O/H_2O (1:10) and HPLC analysis showed complete conversion to **5**, while the HPLC isolated yield was 53% (see the SI). This result with peptide, **2**, clearly defined that terminal or hindered internal tyrosine-containing peptides had little steric or conformation consequences on reactivity, since both the ^{1}H NMR conversion and HPLC isolated yields were substantially the same, in comparison to peptide **1**.

[Tyr^3]-octreotide, **3**, a somatostatin receptor peptide, directly competed phenylalanine, tryptophan, and the disufide linkage, with tyrosine for reaction with excess $[Cp^*Rh(H_2O)_3](OTf)_2$. We found that the dominant reaction product observed was **6** (Chart 1), via HPLC analysis (88% yield), and confirmed by ^{1}H NMR, ^{13}C NMR, 2D NMR, CD, as well as HR-ESI-MS analysis (see the SI). Complex **6** was also found to further react with excess $[Cp^*Rh(H_2O)_3](OTf)_2$ to form a very minor amount of $[(\eta^6\text{-Cp*Rh-Tyr}^3][(\eta^6\text{-Cp*Rh-}^D\text{Trp}^4)]\text{-octreotide}](OTf)_4$, **7** (8% yield, HPLC); the chemoselectivity for tyrosine only reactivity being 92%, complex **6**.

Due to the dynamics of peptides, and their organometallic derivatives, single crystals for X-ray analysis have been difficult to obtain.[2] Thus, the representative structures of both [Tyr^3]-octreotide, **3**, and complex, **6**, were ascertained using 2D NMR techniques, and are shown in Figure 1. To our knowledge, this is the first organometallic-peptide complex to be structurally characterized utilizing this 2D NMR spectroscopic technique.

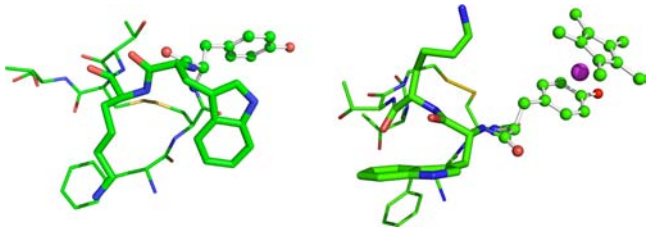


Figure 1. Representative 2D NMR structures of **3** (left), and **6** (right; OTf_2 anions are not shown).

The 2D NMR spectra of both peptide, **3**, and complex, **6**, showed a high number of cross peaks that were well dispersed (2D ROESY, SI). This indicated a stable fold of these peptides, most likely, as a result of the disulfide bond between Cys^2 and Cys^7.[10] In addition, unambiguous NOEs between the aromatic side chain protons of Tyr^3 and the Cp*Rh moiety could be observed for $[(\eta^6\text{-Cp*Rh-Tyr}^3)\text{-octreotide}](OTf)_2$, **6**. Three-dimensional structure calculations were facilitated by the high number of ROE-based distance constraints, that could be extracted from the two-dimensional homonuclear ROESY spectra.[11] Structural parameters of the Cp*-Rh-Tyr moiety have been obtained utilizing X-ray crystal structures of similar η^6-Cp*Rh-phenol complexes.[12] The structures of **3** and **6** were then calculated using simulated annealing protocols, which led to an ensemble of structures (SI). All of the experimental distance and dihedral constraints were fulfilled in both structures, and the precision of both structures was very high (Figure 1). In fact, due to the iterative procedure of the spectral analysis and structure calculations, the ensemble structures of **3** and **6** were characterized by a comparably low heavy atom root-mean-square deviation of approximately 0.39 Å and 0.54 Å, respectively (Figure 1, and SI).

Furthermore, both **3** and **6** adopted the backbone canonical β-turn structure at the well-defined ^{D}Trp^4-Lys^5-dipeptide,

dx.doi.org/10.1021/ja303010k | J. Am. Chem. Soc. 2012, 134, 10321−10324

although their pharmacophore conformations were found to be dramatically different. In the structure of complex **6**, the Cp*Rh group was coordinated to the side chain of Tyr^3 in an η^6 bonding mode, and consequently, pushed the side chain of the $^{D}Trp^4$ residue away, and rotated toward the direction of the Lys^5 residue (Figures 1 and 2). This resulted in the Lys^5 residue being flipped to the other side of the pharmacophore (Figures 1 and 2, and SI). The overall structural similarities of the peptide backbones, demonstrated by 2D NMR, were also confirmed by the CD spectra of [Tyr^3]-octreotide, **3**, with minima at 196 and 218 nm, and a maximum at 230 nm, corresponding to an antiparallel pleated β-sheet and type II′ β-turn, while complex **6** had two less intense, and slightly red-shifted minima at 198 and 221 nm, and no maximum around 230 nm (SI).

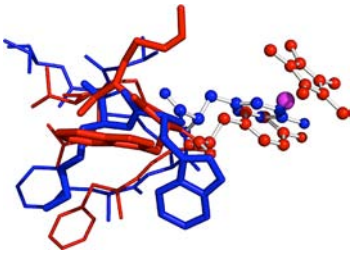


Figure 2. Superimposed 2D NMR structures of peptide **3** (blue) and complex **6** (red); Rh atom (magenta).

The plausible reason for the high chemoselectivity of the η^6-Cp*Rh-tyrosine product with peptides, **1**−**3**, at pH ∼5−6, might be predicated on the dramatic lowering of the pK_a of the phenol hydrogen as the η^2/η^4-Cp*Rh-tyrosine intermediates transition to the final η^6-Cp*Rh-tyrosine complex; i.e., the back donation into the Rh d-orbitals from increased electron density into the phenol aromatic ring as the pH was raised from ∼3 to ∼5−6, provided a driving force for higher reactivity. Moreover, the pH also appears to have controlled the lack of reactivity of all the amino groups via protonation.

The biological consequences of the η^6-Cp*Rh modification of tyrosine-containing peptides, **1** and **3**, were of interest; therefore, our initial bioassay studies were conducted with [(η^6-Cp*Rh-Tyr^1)-leu-enkephalin](OTf)$_2$, **4** (SI). The EC_{50} receptor binding value for [Tyr^1]-leu-enkephalin, **1**, an opioid receptor peptide, and that for complex **4**, at the μ-opioid receptor (MOR), or ∂-opioid receptor (DOR), can be found in Table 1.[13a] It was shown that [(η^6-Cp*Rh-Tyr^1)]-leu-enkephalin](OTf)$_2$, **4**, was an agonist, with nM potency, on cells expressing MOR or DOR alone, as well as on cells co-expressing both MOR and DOR (Table 1 and SI). Thus, η^6-coordination of Cp*Rh to the tyrosine residue of [Tyr^1]-leu-enkephalin, **4**, lowered its potency toward cells expressing only MOR or DOR, but had a similar potency as **1** for cells co-expressing both MOR and DOR, which coincided with previous findings of distinctly different pharmacological profiles for co-expressed versus receptor cells alone, and complements the results with peptide **1**.[13b]

Moreover, a competitive binding experiment was performed on the GPCR somatostatin receptor, rat SST_2, with positive tumoral acinar pancreatic cells (AR42J), using [^{111}In-DTPA,^{D}Phe^1]-octreotide, as a radiotracer. Thus, from these results, [(η^6-Cp*Rh)-Tyr^3)-octreotide](OTf)$_2$, **6**, has a very similar affinity for the SST_2 receptor in comparison to [DTPA,^{D}Phe^1]-octreotide (Table 1).[14] Apparently, the

Table 1. Comparison of the GPCR Receptor Binding and Cancer Cell Growth Inhibition Activity of [(η^6-Cp*Rh-Tyr^1)-leu-enkephalin](OTf)$_2$, 4, and [(η^6-Cp*Rh-Tyr^3)-octreotide](OTf)$_2$, 6, with Peptides, 1 and 3

	substrate			
receptor	**4**	**6**	**1**	**3**
	Binding (nM)			
SST_2[a] (IC_{50})	–	15.8	–	13.8
MOR[b] (EC_{50})	93.3	–	14.3	–
DOR[c]	15.6	–	4.4	–
MOR+DOR	3.4	–	3.3	–
	IC_{50} Growth Inhibition Values (μM)			
MCF7	4.1	4.6	2.8	4.6
HT29	4.6	5.3	3.8	4.3

[a]Somatostatin receptor. [b]μ-Opioid receptor. [c]∂-Opioid receptor.

Cp*Rh moiety η^6-bonded to the tyrosine residue, which was on the opposite side to the $^{D}Trp^4$-Lys^5-pharmacophore (Figure 2), had little effect on the binding of **6** to the GPCR SST_2 receptor. This result suggested that the tyrosine residue was not an important component in the molecular recognition process, at the SST_2 receptor site, and intimated that the flexibility of the receptor site can accommodate major changes in the pharmacophore conformation, but still retain bioactivity. However, the affinity of **6** for the SST_2 receptor, which has been shown to be the most abundant SST subtype in human tumors expressing the somatostatin receptors,[15] was also found to be in the range of other somatostatin peptides.[16]

In other bioassay experiments, the consequence of this Cp*Rh modification of peptides **1** and **3** on the *in vitro* growth inhibition of the breast adenocarcinoma cell line (MCF7) and the human colon carcinoma (HT29) cell line was determined.[17] For these bioassays, both [(η^6-Cp*Rh-Tyr^1)-leu-enkephalin](OTf)$_2$, **4**, and [(η^6-Cp*Rh-Tyr^3)-octreotide]-OTf)$_2$, **6**, were tested and compared to peptides **1** and **3** (Table 1). Interestingly, both **4** and **6** had very similar IC_{50} values with those of peptides **1** and **3**, for both cell lines (Table 1). Since the MCF7 and HT29 cell lines were found to express opioid[18] and somatostatin receptors,[19] among others, this growth inhibition activity observed for both Cp*Rh-peptide complexes, **4** and **6**, might be directly related to their GPCR binding regimes.[20] These latter bioassay studies also confirmed the findings of the GPCR binding experiments, in that, the antiproliferative activity of peptides **1** and **3** was still retained by the Cp*Rh modification of their tyrosine residues, complexes **4** and **6**, and to reiterate, provided information on the potential role of the opioid and somatostatin receptors in the growth inhibition of MCF7 and HT29 cancer cells.

In conclusion, we have shown that reactions of [Cp*Rh-(H_2O)$_3$](OTf)$_2$ with representative GPCR peptides, **1**−**3**, in water, were highly chemoselective for the tyrosine residue, and that this facile bioconjugation technique was pH dependent, while eliminating the necessity for prolonged, stringent synthetic procedures. We also demonstrated the lack of steric and conformational effects for this Cp*Rh peptide modification, since the position of the tyrosine residue, either terminal, **1**, internal, **2**, or on a cyclic peptide, **3**, with proximal phenyl, indole, or an S−S linkage, did not apparently affect reactivity; complex **6** also represented the first reported 2D NMR structure of an organometallic-peptide complex, and retained the canonical β-turn structure at the well-defined $^{D}Trp^4$-Lys^5-dipeptide pharmacophore; however, the conformation of **6** was

dx.doi.org/10.1021/ja303010k | J. Am. Chem. Soc. 2012, 134, 10321−10324

quite different in comparison to peptide **3**. More importantly, we also clearly demonstrated the bioactivity of complexes **4** and **6**, in GPCR binding studies to the opioid and somatostatin receptors, as well as their growth inhibition activity to several cancer cell lines, which were also possibly related to their GPCR binding regimes.

We intend to further attempt to understand the receptor binding phenomena, by conducting future computer docking experiments with complex **4**, utilizing the recently published X-ray structural data for μ-opioid GPCR, which was found to be a homodimer with a co-crystallized morphine analog.[21] We will also extend this bioconjugation technique to other GPCR tyrosine-containing peptides, for potential organometallic-peptide drugs,[22] including tyrosine kinase enzyme inhibitors,[23] and the possible use of *in-cell* NMR techniques to detect the Cp*Rh resonance of the peptide complexes in cell components, and their metabolic pathways.[24] Finally, our reported studies have established the field of bioconjugation of organometallic aqua complexes to GPCR peptides, while GPCRs have been categorized as a superfamily of membrane proteins that regulate all aspects of normal physiology, and thus, are a major target for disease therapy.[4] Therefore, we envision a paradigm shift for the utilization of organometallic aqua complexes, like $[Cp^*Rh(H_2O)_3](OTf)_2$, for pH dependent, chemoselective labeling of peptide aromatic amino acid residues, probing peptide conformations via 2D NMR methods, studying binding regimes to GPCR sites, and understanding their biological mode of action.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis, ^{1}H and ^{13}C NMR, 2D NMR, HR-ESI-MS, HPLC, and CD data for η^6-Cp*Rh-peptide complexes, **4**−**6**, and bioassay procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

R.H.F. wishes to thank the Research Department for Interfacial Systems Chemistry, Ruhr University Bochum, for a Visiting Professorship, Spring 2010, where these studies were initiated, and the Department of Energy under Contract No. DE AC02-05CH11231. N.M-N. and R.S. wish to thank the German Science Foundation, DFG, research unit FOR630 and SFB642, respectively, for support. We also thank Martin Gartmann for NMR expertise; Annegret Knüfer for cell viability studies; Marina Wotske, Ruhr Univ., for HR-ESI-MS; and Gerben M. Franssen, Radboud Univ., for SST_2 cell cultures.

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