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Arene-selective peptide and protein derivatization

Douglas B. Grotjahn *

Department of Chemistry, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182-1030, USA

Contents

Abstract

Chelate complexes of Ru(II) (**12a,b**) featuring an η^5-C_5,η^1-N-$C_5H_4(CH_2)_nNH_2$ ligand ($n = 2$, 3) have been synthesized as arene-selective protein modification reagents. The new chelates produce η^6-arene complexes from a wide variety of arenes, from 1,4-di-*t*-butylbenzene to phenylalanine-containing peptides and proteins. In methanol or nitromethane at room temperature (r.t.), kinetic products are formed by coordination of the Ru center to N- and S-donors (primary amines, histidine, methionine, cystine), but warming to 60°C leads to migration of the Ru center to a phenylalanine ring, with the formation of η^6-arene complexes as robust thermodynamic products. Notably, in water the complex **12a** reacts at r.t. with the 27-residue protein secretin, which contains a phenylalanine residue, to give an η^6-arene complex, which can be purified by HPLC and analyzed by electrospray and MALDI-MS.

* Fax: +1-619-594-4634.
E-mail address: grotjahn@sundown.sdsu.edu (D.B. Grotjahn)

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1. Introduction

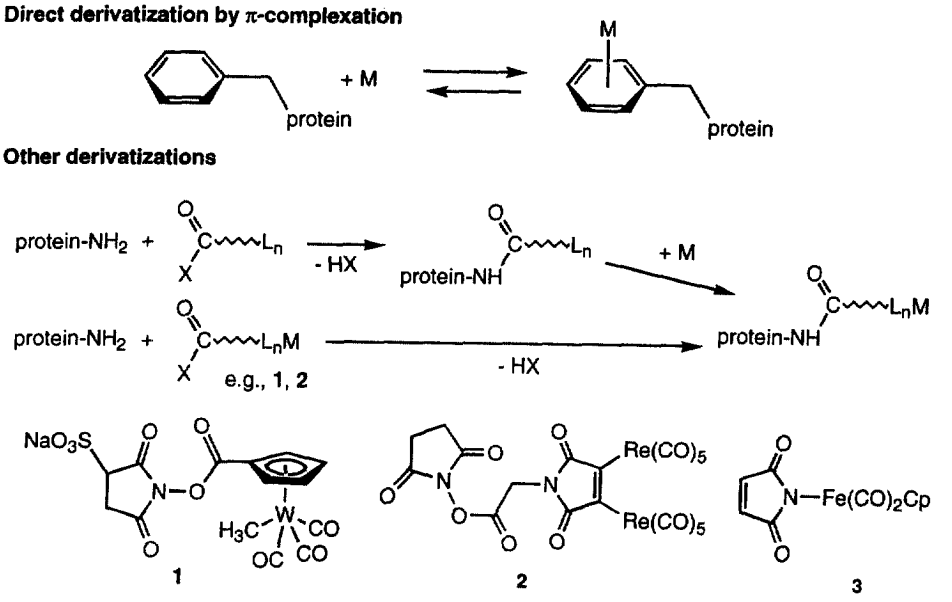
Special properties of organometallic complexes have been used in various ways to study molecules of biological significance. For example, the coordination chemistry of the Cp*Rh fragment and nucleotides in water leads to cyclic structures capable of acting as hosts for aromatic guests [1]. Homolytic cleavage of metal–carbon bonds in organometallics has been used to cleave DNA [2,3]. Organometallic complexes of estradiol have been synthesized as potential radiopharmaceuticals [4,5], and tagging of antibodies with metal carbonyl reagents is the basis of immunoassay procedures [5]. Peptides with *N*-ferrocenoyl substituents have been proposed as structural probes [6], and the unnatural amino acid ruthenocenylalanine has been studied as a pancreatic imaging agent [7]. Radioactive technetium has been attached to the oxygen of *N*-acylated amino acid carboxylates using a cyclopentadienyl moiety [8]. A novel synthesis of the ion $Tc(OH_2)(CO)_3^+$, promising as a reagent for derivatizing biomolecules, was recently reported [9]. Both enzymes [10] and sugars [11,12] have been connected to organometallic fragments. One recent comprehensive review [13] covers the organometallic chemistry of α-amino acids and peptides, whereas another focuses on the uses of CpRu and Cp*Ru complexes of such molecules [14].

In several projects, my group is interested in applying organometallic and coordination chemistry to protein and peptide derivatization [15] and cleavage and stereoselective transformations of organometallic amino acid complexes [16–20]. Site-specific modification of proteins leads to invaluable structural and reactivity information for biochemical and medicinal studies and to new biotherapeutics [21]. Modification frequently relies on the reaction of electrophilic reagents with nucleophilic atom(s) of a protein [21], for example reaction of BrCN with methionine or cysteine sulfur atoms [22a], of isothiocyanates with amino groups [22b], and of the electrophilic halogen sources with the electron-rich π-system of tryptophan [22c]. The organic reagents which react with tryptophan or tyrosine react more slowly or not at all with phenylalanine, although a promising method of iodination in organic solvent was recently reported [23]. The same electrophilic or oxidizing reagents can enter into unwanted side-reactions with nucleophilic S- or N-donors.

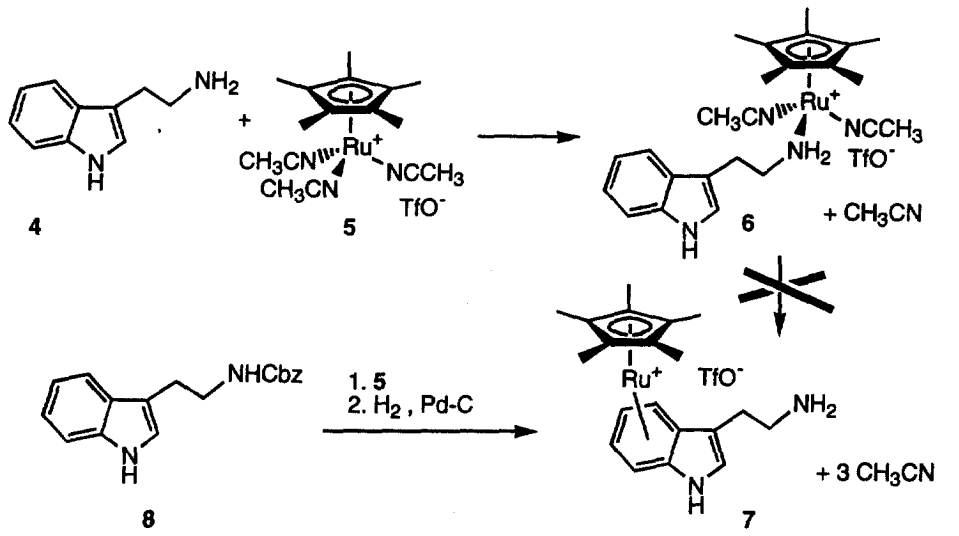
Direct derivatization of phenylalanine or other aromatic residues by π-complexation (top of Scheme 1) could offer a gentle and reversible way to alter proteins. In contrast, thus far, organometallic complexes have been attached to proteins indirectly [24–30], by using the organic moieties of electrophilic reagents such as **1** [26], **2** [28], and **3** [27,29], which react with amino or thiol groups in the protein. Coordination compounds of proteins have also been created by covalent [31,32] or non-covalent [33] attachment of a ligand to a protein, followed by metallation. Direct derivatization by π-complexation could be simpler and reversible, and would represent a new metal–protein interaction [34] which could be used to direct subsequent chemistry to sites near the π-complex.

$CpRu^+$ and Cp^*Ru^+ fragments were attractive candidates for selective protein modification because their η^6-arene complexes are exceptionally stable [14,35]. For example, it is reported that $[CpRu(toluene)]^+$ is oxidized to the corresponding η^6-benzoic acid complex in boiling aqueous $KMnO_4$ [36]. Unprotected phenylalanine (Phe) and tryptophan (Trp) and their dipeptides were converted to Cp^*Ru^+ complexes in non-aqueous solvents [37], and *N*-acylated aromatic amino acids were coordinated to $CpRu^+$ in dichloroethane [38]. In addition, several groups have performed functional group manipulations such as ester- and amide-bond forming reactions on arene complexes of Ru [39–42]. Removal of $CpRu^+$ and Cp^*Ru^+ fragments from arenes is accomplished by irradiation with light in acetonitrile [43–46], which regenerates a tris(acetonitrile) complex that can be recycled for use in subsequent reactions. This procedure seems to work well for all but extremely electron-poor arenes [47]. The only $CpRu^+$ and Cp^*Ru^+ arene complexes which appear to be unstable in the absence of light are those of phenol [48] and of tyrosine [38], perhaps because of facile deprotonation to the neutral oxo-dienyl complexes.

Unfortunately, although the η^6-arene complexes of $CpRu^+$ are quite stable once formed, there were strong indications that good sigma donors such as thioethers, amines, and imidazoles might preempt the π-complexation we desired. For example (Scheme 2), the only product observed from the reaction of tryptamine (**4**) and $Cp^*Ru(CH_3CN)_3^+$ (**5**) was η^1-N complex **6**. The authors reported that all attempts to convert it to η^6-complex **7** failed [49], despite the stability of the latter species, made by uneventful coordination to *N*-acylated tryptamine **8** followed by removal of the Cbz group. Further evidence of the interference of other coordinating groups

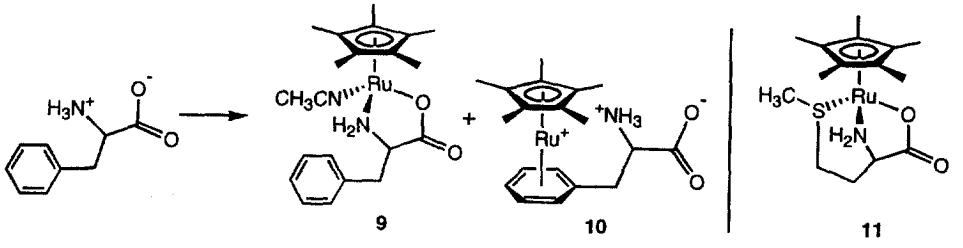


Scheme 1.



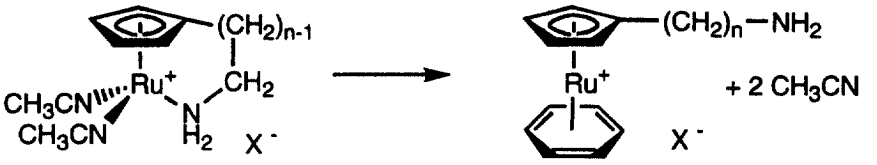
Scheme 2.

comes from the work of Sheldrick and Gleichmann [50] (Scheme 3), who showed that $(Cp^*RuCl_2)_2$ and Phe react in methanol in the presence of NaOMe to give exclusively *N,O*-chelate **9** at 0°C, but a mixture of **9** and η^6-arene complex **10** in a ratio of 1:9 after 3 h under reflux. With methionine, tridentate complex **11** was formed. Moreover, the strong binding of the $Ru(II)(NH_3)_5$ fragment to histidines has been used to install redox-active metal centers in proteins [51]. On the other hand, studies by Fish and coworkers [52–54] showed that Cp- and $Cp^*Ru(CH_3CN)_3^+$ and pyridine derivatives tended to give *N*-coordinated complexes as kinetic products, which isomerized in an intramolecular manner to π-coordinated pyridine complexes [55]. Diphenylacetylene and phenol also gave η^6-arene complexes [48], although the latter ligand becomes very acidic and the conjugate base seems to have limited stability to air. Uneventful complexation to *N*-acylated amino acid derivatives of Tyr, Phe, and Trp was reported [38], although here too the phenolic Tyr complex is unstable. In sum, the available literature thus suggested that we could definitely expect formation of σ-donor complexes as kinetic products, which might or might not be convertible to η^6-arene derivatives.



Scheme 3.

So far, η^6-arene complexes have been reported for $CpRu^+$ and Cp^*Ru^+ fragments, which feature unfunctionalized Cp rings. For the short-range goal of direct protein derivatization, either of these fragments would suffice. However, if one wants to perform other chemistry near the ruthenium center, such as protein cross-linking or directed cleavage of the peptide backbone, the presence of an additional functional group on the periphery of the complex would be desirable. Therefore, chelate complexes **12** were designed. In **12**, the nucleophilic and basic properties of the amine function are attenuated by coordination to the metal, and the amine could be said to be locked. In contrast, if arene coordination succeeded in breaking the amine–metal and acetonitrile–metal bonds, in the resulting η^6-arene protein complex **13** the amine would be an unlocked nucleophile and base. Cyclopentadienyl ligands featuring (*N*,*N*-dialkylamino)- and (*N*-monoalkylamino)alkyl side chains have attracted attention for their unusual properties [56], but with few exceptions the role of the amino group is simply that of hemilabile ligand. However, the ability of an uncoordinated amino function to be protonated can lead to altered reactivity and water solubility. For example, whereas group IV (aminoalkyl)metallocene derivatives with a neutral side chain are water- and air-sensitive, those with a protonated amino function are stable to water and air [57]. Furthermore, thorough comparison of dimeric complexes **14** showed that initial photodissociation was similar for **14a** and **14b**, but that subsequent proton-transfer reactions facilitated by the side chain in **14b** led to very different chemistry [58,59].

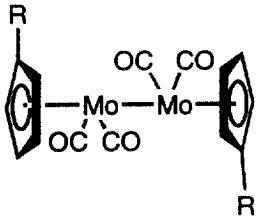


12 a n = 2
b n = 3

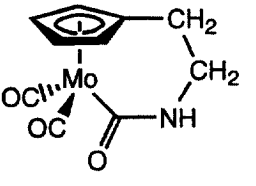
13

With very few exceptions [58–63], studies of aminoalkyl complexes have focused on *N*,*N*-dialkyl compounds. *N*-Monoalkyl analogues can behave similarly, but chelates containing an amido ligand on Re [61,62] and Ti [63] have been reported. Attack of an unsubstituted amino group on a CO ligand gave **15** [59].

In sum, we expected that aminoalkyl-substituted Ru complexes **12** and **13** would show improved water solubility and different chemistry than the CpRu and Cp*Ru compounds used by others.



14 a R = H
b R = $CH_2CH_2NH_3^+$



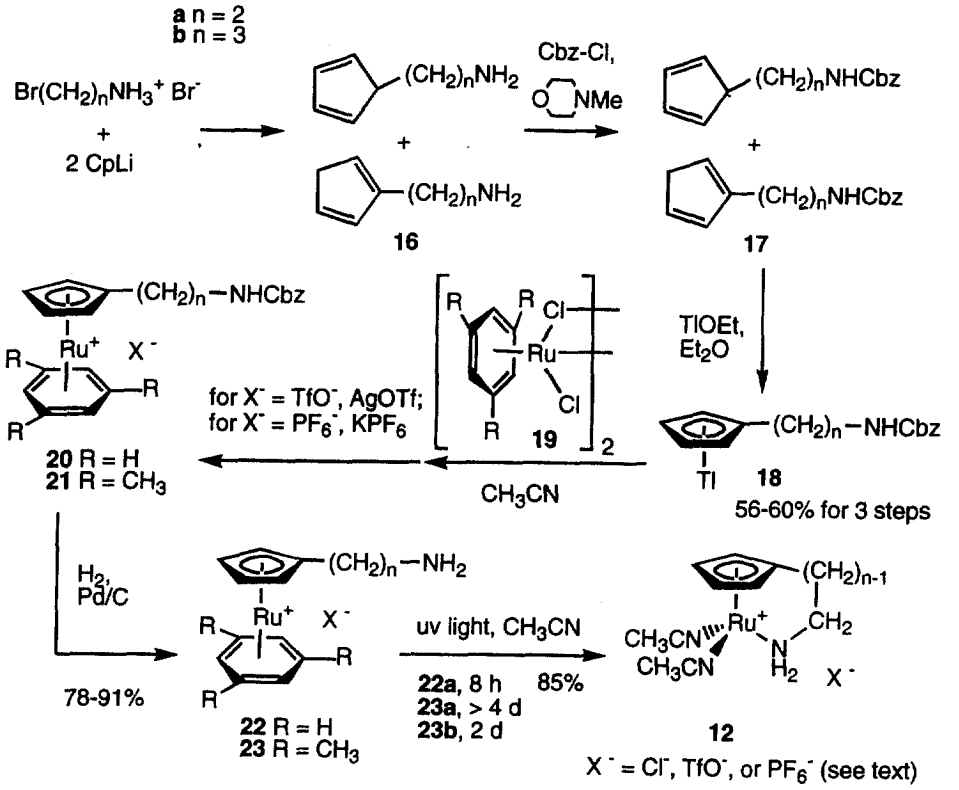
15

The challenges to be met in this project were:

1. Develop an efficient synthesis of chelate complexes **12**.
2. Show that the chelate in **12** can be broken by arene complexation to the Ru center.
3. Show that arene complexation can be achieved in the presence of amines, carboxylic acids, methionine, histidine, cystine, and cysteine.
4. Demonstrate the ability to use **12** in water.
5. Show that a protein–Ru complex (**13**) can be formed, purified, and characterized.

2. Synthesis of chelate complexes

The synthetic route chosen for the chelate complexes (Scheme 4) started with the introduction of the aminoalkyl side chain on cyclopentadiene, followed by nitrogen protection. Conditions used by others were modified for the alkylation reaction using salts $Br(CH_2)_nNH_3^+Br^-$ [61,64,65]. CpLi (1 mol as base, 1 mol as nucle-



Scheme 4.

ophile) was used. The crude alkylated products **16**, presumed to be a mixture of diene isomers, were extracted into ether and the amino group was protected using carbobenzyloxy chloride (Cbz–Cl) and a tertiary amine base. After aqueous washing to remove by-products, TlOEt [66] was added to the ethereal solution of crude isomers **17**, resulting in precipitation of powdery Tl salts **18**. These compounds were characterized by IR, ^{1}H, and $^{13}C\{^{1}H\}$ NMR spectroscopies before being used in the next synthetic step. The yields of salts **18** (56–60%) compare favorably with the 20% overall yield of **16** obtained previously in four steps starting from CpH and oxirane [58,67,68].

The remainder of the synthesis was inspired by the original report of Gill and Mann [42]. In acetonitrile the Tl salts **18** and 0.5 mol of dimer **19** (R = H or CH_3) were allowed to react for 5 h before TlCl was removed by filtration. The resulting chloride salts **20**–Cl and **21**–Cl were soluble in water. Depending on the solubility characteristics desired for the final chelate complexes, the chloride counterion was exchanged for either triflate or hexafluorophosphate. In the chloride and triflate cases **20a**–Cl and **20a**–OTf, careful purification of intermediate **20a**–Cl by recrystallization was important to obtain clean samples of chelate complexes **12a**–Cl and **12a**–OTf. In contrast, the higher solubility of hexafluorophosphates in organic solvents (even CH_2Cl_2!) could be used to advantage: addition of KPF_6 to aqueous solutions of the crude chloride salts resulted in precipitates which were fully extracted with CH_2Cl_2 to afford intermediates **20a**–PF_6, **21a**–PF_6, and **21b**–PF_6. The triflate **20a**–OTf was obtained in 95% yield from the corresponding purified chloride salt by treatment with AgOTf. Removal of the Cbz group from all salts was accomplished readily in 78–91% yields by catalytic hydrogenation over Pd on carbon in methanol [49]. In earlier attempts at this step, it appeared that less-pure starting materials required much higher catalyst loadings or longer reaction times for completion, suggesting some catalyst poisoning. The *N*-protection and deprotection sequence was necessary, because although a Tl salt could be made from **16**, it reacted much more slowly with **19** and seemed to produce a mixture of products, which we presume to involve varying degrees of Tl-coordination to the unacylated amino function.

The final step in the synthesis involved removal of the arene by irradiation of the sandwich complexes with Pyrex-filtered UV light in CH_3CN [43–46]. Qualitatively, the time required for completion of the reaction strongly depended on the size of the arene and the length of the side chain, the reaction being slowest for the larger arene and for the shorter side chain. Mann et al. proposed that light aids the removal of arenes from CpRu-η^6-arene complexes by weakening the metal–arene bonds, creating a distorted but still-coordinated arene ligand, and that the nucleophilicity of the solvent plays a role in a subsequent step [45]. As explained below, the amino ligand of the chelate **12** with the shorter side chain was displaced by arenes about twice as fast, so we tentatively suggest that the chelate ring in **12a** is more strained than that in **12b**, and that assistance by the amino group of **23a** is more difficult than by the amino moiety of **23b**.

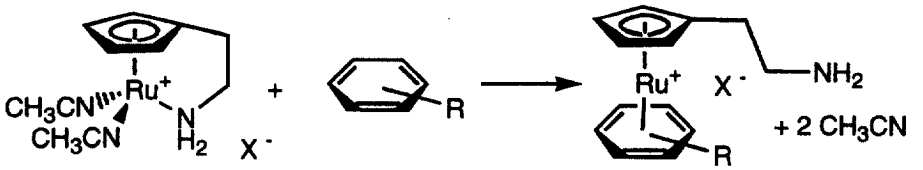
The ^{1}H NMR spectral data for the methylene protons adjacent to the amino function show interesting trends. For the $-CH_2NH_2$ of the two-carbon side chain, the chemical shift of the triplet changes from δ 2.71 ppm in **22a** to 3.64 ppm in **12a**; a similar downfield shift on chelation has been seen for 1-(*N*,*N*-dimethylamino)ethyl-2,3,4,5-cyclopentadienyl complexes of Rh and Ir [69] and (aminoethyl)cyclopentadienyl complexes of Mo [58,59]. Chelation is indirectly supported by ^{1}H NMR data for the two (not three) CH_3CN ligands (s, δ 2.39 ppm, 6 H) and combustion data. Surprisingly, for the unchelated and chelated ligands in **23b** and **12b**, the chemical shifts of the methylene protons next to the amino group were almost identical (for **23b**, 2.71, for **12b**, 2.75 ppm), but chelation could be indirectly inferred from the six-proton singlet at δ 2.37 ppm for two CH_3CN ligands.

3. Binding to arenes

Although it is well-known that $[CpRu(CH_3CN)_3]^+$ derivatives react readily with arenes, it was not clear if chelates **12a,b** would undergo the same reaction, because of the presence of the amino ligand in the coordination sphere. Particularly worrisome was the literature observation that $Cp^*Ru(CH_3CN)_3]^+TfO^-$ and the basic nitrogen of tryptamine led to amine complex **6** (Scheme 2), which decomposed rather than producing the expected η^6-arene complex **7** [49].

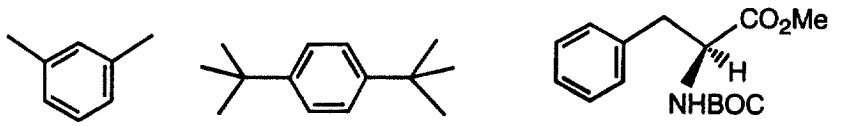
Gratifyingly, dissolution of arenes **24** (Scheme 5) and chelates **12a**–PF_6 or **12b**–PF_6 in CD_3NO_2 or CD_3OD led to corresponding arene complexes **25**. At ambient temperature, **12a** required about 2 days to react completely, whereas at 60°C reaction was complete within 1–6 h. Surprisingly, the bulk of arene substituents did not dramatically effect the time required for completion, as seen by successful syntheses of **25a** and **25b**. Formation of arene complexes was implicated by several telling spectral changes. For example, in 1,3-dimethylbenzene complex **25a**, the arene ring protons appear between 6.04 and 6.14 ppm, and the corresponding carbons between 80 and 105 ppm, both indicative of π-complexation to the Ru center. Meanwhile, whereas in chelate **12a** the protons of the cyclopentadienyl ring appear at 3.94 and 4.24 ppm (two t, $J = 1.8$ Hz), in arene complex **25a** the resonances show up at 5.22 and 5.31 ppm (two t, $J = 1.6$ Hz), consistent with replacement of the three N donors in **12a** by the π-acidic arene in **25a**. Finally, the $-CH_2NH_2$ protons in **12a** and **25a** resonate at 3.64 and 2.84 ppm, respectively, showing the existence of an unchelated side chain in the final product.

Compounds **24a–g** behaved similarly, showing the tolerance of the complexation reaction toward carboxylic acids (**25e**, **25f**) and the ability to use an unprotected amino acid (L–Phe–OH, **25d**). Ultimately, the ability to make arene complexes from the other substrates in Scheme 5 (**24h–24k**) was shown, but only after identification of some intermediates in the complexation process.

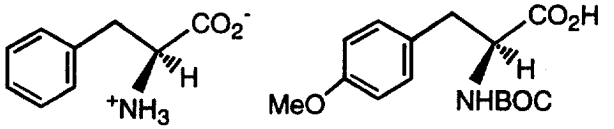


12a arene **24** η^6-arene complex **25**

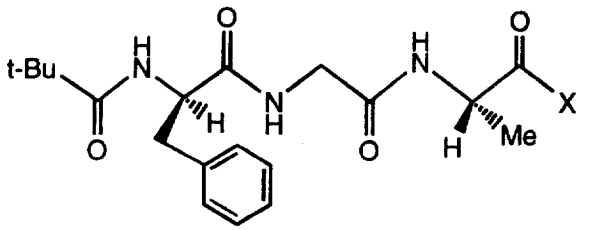
Arenes used:

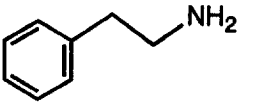


a **b** **c** BOC-L-Phe-OMe



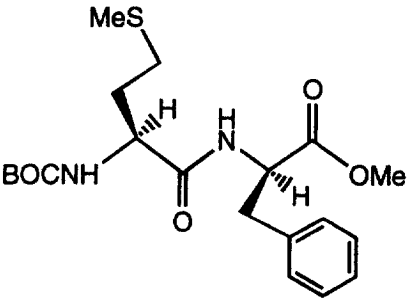
d L-Phe-OH **e** BOC-L-Tyr(OMe)-OH

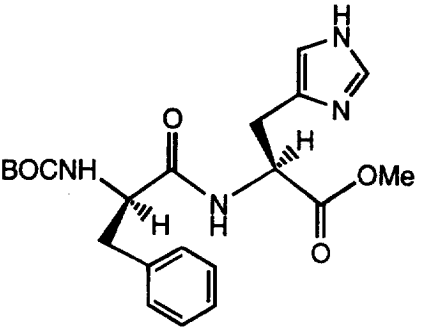




f X = OH (Piv-D-Phe-Gly-L-Ala-OH)
g X = NH_2 (Piv-D-Phe-Gly-L-Ala-NH_2)

h





i BOC-L-Met-D-Phe-OMe **j** BOC-D-Phe-L-His-OMe

k synthetic human secretin =
His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Glu-Gly-Ala-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH_2

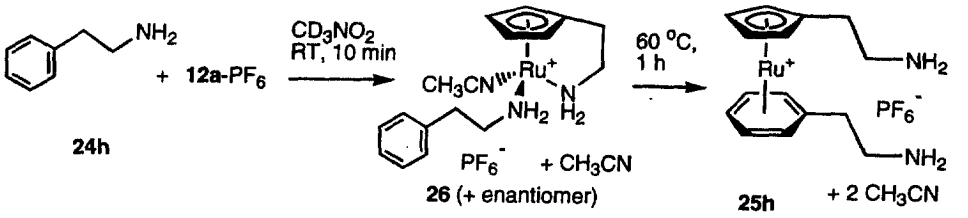
Scheme 5.

4. Arene coordination in the presence of other ligands—scope and limitations

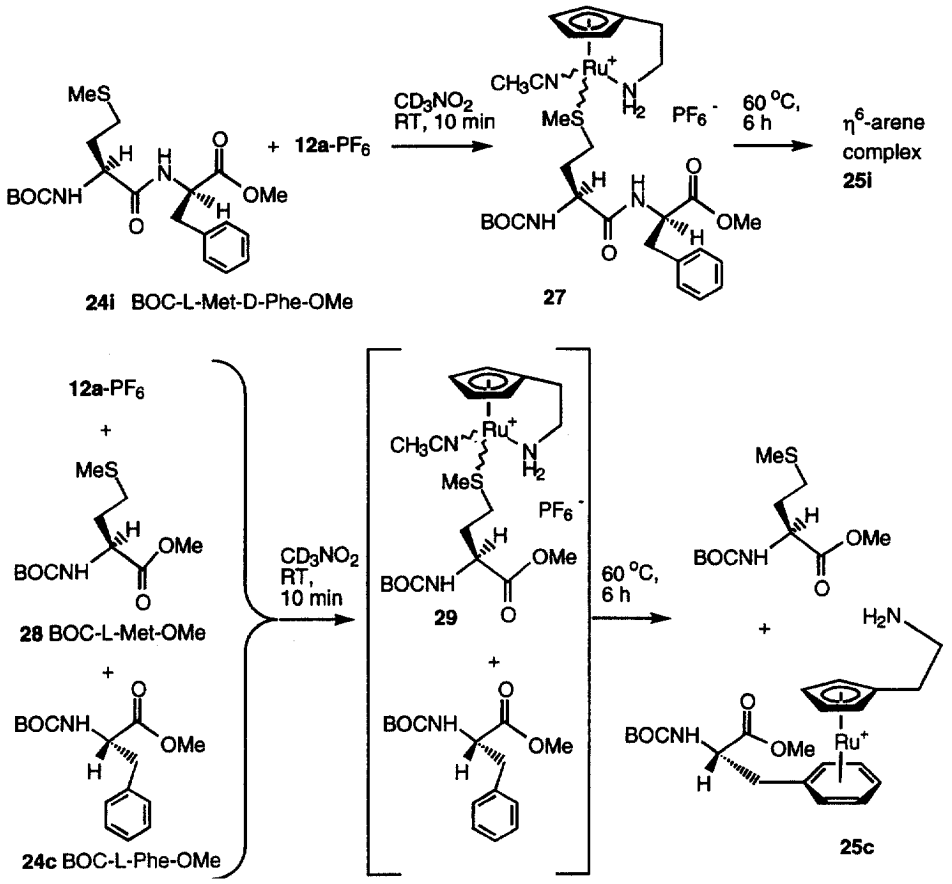
On the basis of published results obtained using $CpRu(CH_3CN)_3^+$ and its Cp* analog (see Section 1), the formation of σ-complexes as kinetic products could be expected in reactions of **12a** with polyfunctional arenes bearing σ-donors. Our task was to characterize such intermediates and then see if eventually arene π-complexes **25** would form as thermodynamic products.

Because the coordination of the amine function in **12a** and **12b** was successfully broken on exposure of the complexes to arenes, phenylethanamine (**24h**) was chosen as an initial test of functional group interference to arene binding. Compound **12a** was gone 10 min after phenylethanamine and **12a**–PF_6 were mixed at ambient temperature in CD_3NO_2. Some excess arene was still present, but the data for the major species in solution were fully consistent with formation of a chiral intermediate **26** (Scheme 6). Four narrow unresolved multiplets at δ 3.70, 3.81, 4.06 and 4.12 ppm of equal integration were ascribed to the diastereotopic protons of the cyclopentadienyl ring. The fact that these protons resonated near δ 4 ppm, close to the chemical shifts of the ring protons in **12a**, was consistent with maintenance of three nitrogen σ-donors on the metal. Small resonances near 5.4 and 6.2 ppm indicated that some η^6-arene complex(es) had formed, but the small integral associated with these peaks and the large one associated with the multiplet near δ 7 ppm was consistent with identification of **26**, rather than an η^6-arene complex as the major species. Warming the reaction mixture at 60°C for 1 h resulted in η^6-arene complex **25h** and CH_3CN as the only identifiable products, the former easily recognizable by the appearance of two triplets at δ 5.29 and 5.40 ($J = 2.4$ Hz, 2 H each) and a five-proton multiplet between 6.07–6.20 ppm for the protons on the cyclopentadienyl and phenyl rings, respectively. Resonances for the eight methylene protons of the complex appeared as four two-proton triplets between 2.40 and 2.93 ppm.

A similar experiment with tryptamine (**4**) and **12a** in CD_3NO_2 afforded a solution whose NMR spectral data were consistent with the presence of an intermediate η^1-*N*-tryptamine complex. Surprisingly, heating the solution at 60°C for several hours led to decomposition, inferred because the peaks for the (aminoethyl)cyclopentadienyl ligand disappeared altogether while peaks for an indole derivative remained. Further work will be necessary to elucidate the course of this reaction, but we tentatively conclude that in reactions of **4** with **12a** or **5** [49],



Scheme 6.



Scheme 7.

the indole ring is somehow responsible for decomposition of the η^1-amine complexes.

More serious tests of arenophilicity were deemed to be the imidazole of histidine, the thioether of methionine, the disulfide linkage of cysteine, and the thiol group of cystine. Therefore, dipeptides **24i** and **24j** were synthesized. The presence of the N–BOC and methyl ester moieties made the compounds readily soluble in CD_3NO_2. For example, when **24i** and **12a**–PF_6 (Scheme 7) were dissolved in CD_3NO_2 and the resulting mixture was analyzed after 10 min, resonances due to **12a** were gone. The mixture was clearly more complex than the one from phenylethanamine (**24h**), because four singlets near 2.4 ppm, tentatively ascribed to η^1-S complexes **27**, were seen. (Chiral centers in expected intermediate **27** would be at S, Ru, and the homochiral dipeptide backbone, so up to four stereoisomers of **27** could be present.) However, from the relative integrals of a large multiplet at 7.2–7.4 and small, poorly resolved resonances between 5.3 and 6.2 ppm (ratio ca.

3:1) it was clear that any η^6-arene complex(es) was or were minor product(s). Because of the complexity of the spectrum, which included resonances near 4.2 and 4.8 ppm for the α protons of the two amino acid residues [–C*H*(R)NH–], resonances for the diastereotopic cyclopentadienyl protons of the presumed intermediate **27** were not readily identifiable.

Significantly, when the mixture was warmed to 60°C for 6 h, clean formation of η^6-arene complex **25i** occurred, as demonstrated by the appearance of two narrow two-proton multiplets at 5.33 and 5.41 ppm and a five-proton multiplet at 6.13–6.20 ppm. Notice that because of the chiral dipeptide, all cyclopentadienyl and phenyl ring protons and carbons are unique, although for example the chemical shift differences between the cyclopentadienyl protons nearest the ring substituent are small. A three-proton singlet at 2.05 ppm could be ascribed to the uncoordinated CH_3S– group of the methionine side chain.

A similar experiment starting with **24j** showed that the Ru center initially binds to one or more histidines at room temperature (r.t.), but migrates to the Phe ring within 6 h at 60°C to form η^6-arene complex **25j**.

While these results are a spectacular demonstration of the arenophilicity of the Ru fragment, these conversions may only serve as models for proteins in which the strong S- or N-donor is close to Phe, and intramolecular migration of the Ru fragment is feasible. For example, in **27**, the Ru could migrate to the amide carbonyl of the protein backbone, and thence to the aromatic ring of Phe; in other words, the protein may provide hemilabile ligands to assist in movement of the metal center. That methionine need not be closely connected to Phe was shown (Scheme 7) by mixing **12a**–PF_6, BOC–L–Met–OMe (**28**), and BOC–L–Phe–OMe (**24c**) in CD_3NO_2 in a ratio of 1:1:1. Within minutes at ambient temperature, little η^6-arene complex had formed, but most **12a** was gone and the appearance of three new singlets between 2.4 and 2.55 ppm and several narrow multiplets between 4.3 and 4.5 ppm suggested formation of **29**, perhaps as a mixture of diastereomers differing in their configuration at S. In the region 7.2–7.4 ppm, resonances for the five protons of the aromatic ring of free arene **24c** were seen. Warming the mixture at 60°C for 1 h led to the η^6-arene complex of the Phe derivative **25c** and free BOC–L–Met–OMe (**28**). A similar experiment with **24c** and a histidine derivative showed that intramolecular migration from histidine N to the π-system of Phe occurred. Finally, the tolerance of disulfides was shown by a similar experiment conducted with **12a** and unprotected amino acid derivatives cystine and Phe, affording the η^6-Phe complex **25d** after warming of the mixture. Only the thiol group of cysteine seems to prevent formation of an arene complex. However, since there are several procedures for S-oxidation of thiols to the corresponding sulfonic acid cysteic acid [22d], presumably one could first oxidize a protein prior to application of the Ru complex **12a**.

Thus, the model studies show that with the exception of thiols, none of the polar groups found in proteins should prevent achievement of arene complexation to the Ru center of **12a**. However, in order for the chemistry to be practical, we still needed to show that **12a** could be used in water at protein concentrations far lower than the ca. 0.01 M level customary in the NMR tube experiments described so far.

5. Binding to the small protein secretin

The high arenophilicity of the complexes encouraged attempts to react **12a** with the gastrointestinal hormone secretin [70,71] (mass 3039 da). Secretin (**24k**) was chosen because it contains 27 residues, including a unique Phe at position 6. In addition, the ability of the Ru center to bind selectively would be challenged by the presence of His at position 1 (the N-terminus), four Arg, one Asp, and two Glu residues, all containing potentially interfering groups. Synthetic human secretin was obtained from Sigma, and as supplied featured an unnatural C-terminal amide group because of the relative ease of synthesizing such compounds on solid phase. Secretin (0.1 or 0.25 mg) and a known amount of the additive $(CH_3)_3SiCD_2CD_2CO_2Na$ as internal standard for NMR integration were dissolved in an NMR tube in deoxygenated CD_3OD, D_2O, or D_2O containing a 1:1 carbonate–bicarbonate buffer, designed to maintain a pH of 8.3. The ^1H NMR spectra of the resulting solutions were acquired using conditions determined to give accurate integrations (30 s delay between pulses; presaturation of the HOD peak in the case of D_2O solutions was also used). Using this procedure, the amount of protein in the solution could be verified and for the 0.25-mg experiments protein concentration was on the order of 100 μM, roughly 0.01 times the concentration of arene used in the model experiments on amino acid and dipeptide derivatives.

In all secretin binding experiments, after **12a** was added, arene binding was monitored by looking for the disappearance of resonances near 7.2 ppm for the arene protons of Phe, and the appearance of resonances near 6.2 ppm for the Phe ring protons of the $(C_5H_4R)Ru(\eta^6\text{-Phe})$ unit. In earlier experiments in CD_3OD, the NMR tube was warmed at 60°C to complete the complexation, and the yield of η^6-arene complex appeared to be at least 70%. In later experiments in D_2O, yields appeared to be quantitative. After several experiments were conducted to optimize reaction conditions, to a solution of secretin in D_2O containing a carbonate–bicarbonate buffer was added a solution of chelate **12a**–OTf (3.3–10 equivalents) in CD_3OD. The amount of CD_3OD thus added was less than 0.1 ml, so D_2O was the predominant solvent. The triflate analog **12a**–OTf was used in these later experiments because it was more soluble than **12a**–PF_6. Importantly, a remarkable and useful solvent effect was revealed: in D_2O, complete consumption of free arene occurred within 8 h at r.t., very promising conditions for future applications to protein chemistry, where heating of samples is generally undesirable. Although further experiments are needed to determine why water accelerates the complexation reaction, we suspect that it may aid the loss of the amino ligand from the coordination sphere of the ruthenium.

In these experiments, up to ten equivalents of **12a**–OTf were added to complete the consumption of free secretin. However, in the ^1H NMR spectrum of the resulting mixture, there appeared to be several sets of resonances, suggesting that the excess **12a** was involved in binding to several places on the protein, for instance at one of the heterocyclic nitrogens of His, at the N-terminus, and on the unlocked amino group of the ligand itself. The existence of polyruthenated species at this stage was corroborated by observation of MALDI-MS peaks of masses 3353 and

3465, along with a peak of mass 3253, close to the 3247 expected for the most abundant isotopomer of the ion [secretin + $Ru(C_5H_4CH_2CH_2NH_2]^+$.

To remove excess Ru centers from the protein, a relatively odorless thiol, 3-mercaptopropionic acid (MPA) was added to the mixture in a 20-fold excess relative to **12a** used. Within 16 h, the spectrum had simplified considerably, and one set of resonances for imidazole and phenylalanine ring protons was seen. Furthermore, between 5.2 and 5.9 ppm, one major pair of narrow multiplets ascribable to cyclopentadienyl ring protons in **25k** was found. The integrals for the former resonances implied a quantitative yield of **25k**, within the uncertainty of the data.

The contents of the tube were purified by reverse-phase HPLC on a Zorbax C-3 column using undeoxygenated mixtures of trifluoroacetic acid in water and acetonitrile. Using a gradient of increasing acetonitrile-containing solvent, **25k** eluted after 14.6 min, secretin itself eluted after 19.1 min, and a $CpRu^+$–secretin complex prepared from **24k** and $[CpRu(CH_3CN)_3]^+TfO^-$ appeared after 16.0 min. Thus, apparently the increasing positive charge on going from secretin (**24k**) to $CpRu^+$–secretin to **25k** increases polarity and decreases retention time.

The constitution of **25k** was verified in two ways, by mass spectrometry and by automated Edman degradation. Both MALDI and electrospray MS were successful. Because the MALDI-TOF laser used produces pulses of 337 nm light and because CpRu(η^6-benzene)PF_6 exhibits $\lambda_{max} = 325$ nm [46], it is a bit surprising that in most experiments, we found that **25k** and other derivatives **25** could be analyzed successfully. The higher resolution of the electrospray instrument employed allowed determination of the mass of the most abundant isotopomer as 3249.4 da, compared with a value calculated for $C_{137}H_{230}N_{45}O_{40}Ru$ of 3247 da.

The Edman degradation experiment was run for seven cycles. In a control run on secretin itself, the normal HPLC peaks for cleavage products (phenylthiohydantoin or PTH derivatives) of each amino acid were seen; thus, in the sixth cycle, the PTH derivative of phenylalanine was identified by its HPLC peak. However, even when large quantities (ca. 200 pmol) of ruthenated secretin **25k** were subjected to the same degradation, in cycle 6, a peak for the PTH of phenylalanine was undetectable, whereas in cycles 1–5 and 7 the expected PTH compounds were found in good yields. Thus, although no effort was made to find the peak for the presumed PTH of η^6-ruthenated Phe, these results clearly show that Phe had been covalently modified and that the presence of a η^6-ruthenated amino acid residue does not interfere with the degradation.

6. Removal of the arene in water

Because of the small quantities of **25k** generated, arene removal studies were conducted on a model η^6-benzene compound, the chloride and triflate salts **22a**–Cl and **22a**–OTf. Whereas photolysis of **22a**–OTf in an NMR tube in pure CD_3CN was complete within 8 h, similar reactions in D_2O–CD_3CN mixtures required significantly longer times. For ultimate application to proteins, the highest proportion of water was deemed desirable, but as a compromise between solvent compo-

sition and reaction rate, a 1:3 D_2O–CD_3CN mixture was used. After irradiation for 32 h, **22a**–Cl gave benzene in 91% yield, along with what was presumed to be **12a**–Cl. Control experiments involving photolysis of CpRu(η^6-benzene)OTf in CD_3CN and a 1:3 D_2O–CD_3CN mixture showed that changes in solvent hydrogen-bonding to the amino group of **22a** are probably not a factor. Although many factors could be responsible for the reduction in rate in the presence of water, our working hypothesis is that the hydrophobic effect [72] raises the free energy of free benzene as water concentration is increased, and this could raise the free energies of transition states or intermediates on the way to arene loss.

7. Conclusions

Efficient syntheses for chelate complexes **12a,b** with various counterions were developed. In NMR tube experiments at approximately 0.01 M concentrations in methanol or nitromethane solvent, arenes do break the chelate ring of **12a** within 2 days at ambient temperature, or within 1–6 h at 60°C. Polyfunctional arenes such as phenylethanamine and dipeptides containing methionine or histidine in addition to phenylalanine bind initially through the heteroatoms, but form robust η^6-arene complexes under mild conditions, within 6 h at 60°C. It was shown that the migration of the Ru center from a heteroatom to a phenylalanine ring does not depend on proximity of the two ligands in a molecule. In binding **12a** to the histidine- and phenylalanine-containing small protein secretin at about 100 μM concentrations, the use of D_2O as solvent allows η^6-arene complexation to be completed within 8 h at ambient temperature. The ability to form η^6-arene complexes in water at r.t. is very promising for future application to protein chemistry, where heating of samples is generally undesirable. Significantly, complex **12a** and intermediates en route to π-complex **25k** tolerate water as solvent. Finally, standard techniques of protein purification and characterization (mass spectrometry, Edman degradation) can be used on the η^6-arene complex of secretin (**25k**). The beneficial effects of water on the coordination of arenes and the retardation of arene loss by water are phenomena under investigation. The study and application of organometallic compounds in the non-traditional, interdisciplinary areas of aqueous chemistry and biochemistry is expected to lead to unusual and useful results for some time to come.

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