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A Ruthenium(II) Tris(bipyridyl) Amino Acid: Synthesis and Direct Incorporation into an α-Helical Peptide by Solid-Phase Synthesis

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The synthesis of a ruthenium(II) tris(bipyridyl) amino acid is described along with its incorporation into a helix-forming peptide using standard solid-phase peptide synthesis methods. The physical properties of the metalloamino acid separately and in the context of the peptide are reported. The electrochemical potential, absorption spectrum, emission and excitation spectra, and emission lifetime data for the ruthenium(II) tris(bipyridyl) amino acid are similar to those of ruthenium(II) tris(bipyridine), indicating that the amino acid functionality introduced on one of the bipyridine rings does not strongly perturb the properties of the metal complex. The ruthenium(II) tris(bipyridyl) amino acid is amenable to direct incorporation into a synthetic peptide using solid-phase methods and BOC/benzyl chemistry. The 22 amino acid alanine-based peptide produced is 67% helical at 0 °C in aqueous buffer, as measured by circular dichroism spectroscopy. The 2,2,2-trifluorethanol helix induction curve and the temperature dependence of the helicity are typical for alanine-based peptides. The Lifson–Roig helix propagation parameter, *w*, derived from circular dichroism and NMR-monitored kinetic hydrogen–deuterium exchange is 0.5 ± 0.1 at 0 °C, indicating a moderate helix propensity for this metalloamino acid.

Introduction

There is substantial interest in the development of metal-binding amino acids[1−3] and their incorporation into peptides and proteins.[4] These metal-binding amino acids include examples with aminodiacetic acid,[1] phenanthroline,[2] and bipyridine,[3] as side chains. Bidentate side chain ligands are used because of their high metal binding affinity. The insertion of such amino acids, followed by metal incorporation, can affect the structure or stability of the peptide or protein.[1,4c] They can also be used as a preformed metal binding site,[5] similar to the framework provided by proteins for binding metals. Metal-binding amino acids have also been used to specifically attach redox-active metals to proteins, for the study of the electron-transfer process.[4a,b]

In some instances, it is desirable to incorporate two metal binding sites into a peptide in a specific manner,[6] particularly for the study of directional electron transfer. This goal can be accomplished if the metals are added as preformed complexes during solid-phase peptide synthesis. Thus, it would be particularly advantageous to develop a metal-loamino acid which is stable to the standard conditions of solid-phase peptide synthesis. Such an amino acid would allow specific incorporation of metals anywhere in a peptide sequence. To this end, we have synthesized a ruthenium(II) tris(bipyridyl) amino acid.

We recently reported the synthesis of the BOC-protected bipyridine amino acid *N-tert*-butoxycarbonyl-2-amino-3-(4′-methyl-2,2′-bipyridin-4-yl)propanoic acid (**1**),[7] using commercially available 4,4′-dimethyl-2,2′-bipyridine as starting

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material. A significant improvement to this synthesis is presented here. The preparation of the novel ruthenium(II) tris(bipyridyl) amino acid **2** through a reaction of *cis*-dichlorobis(2,2′-bipyridine)ruthenium(II) with **1** is then described. The electrochemical properties of this metalloamino acid, as well as its photophysical properties as a function of solvent and temperature, are described. Solid-phase peptide synthesis is then used to incorporate this novel metalloamino acid into a 22 amino acid alanine-based peptide, expected to form an α-helical structure.[8] The structural properties of this peptide have been analyzed by circular dichroism and NMR-monitored kinetic hydrogen−deuterium exchange methods,[9] providing a direct evaluation of the helix propensity of this ruthenium(II) tris(bipyridyl) amino acid. The photophysical properties of this metalloamino acid in the context of the peptide have also been evaluated.

Experimental Section

Materials. *cis*-Dichlorobis(2,2′-bipyridine)ruthenium(II) dihydrate and ammonium hexafluorophosphate were obtained from Strem Chemical, Inc. CM-sepharose was obtained from Pharmacia (Piscataway, NJ). *p*-Methylbenzhydrylamine (*p*-MBHA)[10] resin, the BOC amino acids, and the BOP coupling reagent were obtained from Calbiochem-NovaBiochem International (La Jolla, CA). The HATU coupling reagent, 2,2,2-trifluoroethanol, *N*-(diphenylmethylene)glycine *tert*-butyl ester, and cesium hydroxide were obtained from Aldrich (Milwaukee, WI). 4-Bromomethyl-4′-methyl-2,2′-bipyridine was prepared as previously described.[11] *O*(9)-Allyl-*N*-9-anthracenylmethylcinchonidium bromide was prepared by the method of Corey et al.[12]

DMSO was distilled from calcium chloride under vacuum and stored over molecular sieves. Toluene was dried by storing it over molecular sieves. DMF was used from a fresh bottle; 3 Å molecular sieves were added to the bottle upon opening.

General Methods. UV/vis spectra were obtained on a Beckman DU640 spectrophotometer. ^{1}H NMR spectra were acquired on a Varian Mercury 400 MHz NMR spectrometer. Elemental analysis was carried out at Galbraith Laboratories (Knoxville, TN). The enantiomeric excess (ee) was determined using a CrownPak(+) column (Daicel), as described previously,[5,7] with 10% methanol in 0.1 M $HClO_4$ as the mobile phase. Optical rotation was determined on a JASCO DIP-4 digital polarimeter, using methanol as solvent. Amino acid analysis and MALDI-TOF mass spectrometry were performed in the laboratory of Dr. John Stewart at the University of Colorado Health Sciences Center. Amino acid analyses were done with a Beckman 6300 amino acid analyzer calibrated with an amino acid standard sample. An authentic sample of the ruthenium amino acid was used to calibrate for its presence in the amino acid analysis of the metallopeptide. MALDI-TOF mass spectra were obtained using α-cyanocinnamic acid as the matrix. Electrospray mass spectrometry was done at Macromolecular Resources, Fort Collins, CO. Electrochemical measurements of the amino acid were performed in the laboratory of Professor C. Michael Elliot at Colorado State University. The metalated amino acid is a PF_6^- salt, so the potentials were measured in either 0.1 M $TBAPF_6$/acetonitrile or a 1:1 mixture of water/acetonitrile. The potentials were measured relative to an SSCE reference electrode (0.2415 V vs NHE) or against ferrocene (0.307 V vs SCE) as an internal standard. The concentration was approximately 2 mg/5 mL. In all experiments, the ruthenium amino acid **2** and peptide concentrations were calculated using the extinction coefficient of the absorption band at 455 nm determined for compound **2**.

Synthesis of *N*-(Diphenylmethylene)-2-amino-3-(4′-methyl-2,2′-bipyridin-4-yl)propanoic Acid *tert*-Butyl Ester (5). *N*-(Diphenylmethylene)glycine *tert*-butyl ester (295 mg, 1 mmol) and the phase-transfer catalyst *O*(9)-allyl-*N*-9-anthracenylcinchonidinium bromide (57 mg, 0.1 mmol) were dissolved in dichloromethane (10 mL). 4-(Bromomethyl)-4′-methyl-2,2′-bipyridine (397 mg, 1.5 mmol) was dissolved in 1 mL of dichloromethane and added to the reaction mixture. The reaction mixture was cooled to −78 °C with a dry ice/acetone bath. Cesium hydroxide (1.68 g, 10 mmol) was then added, and the reaction mixture was stirred for 24 h. The organic phase was washed with water (3 × 10 mL) to remove CsOH. The solvent was removed, leaving a brown oil. The crude product was purified on a silica column, with 5:1 hexanes/ethyl acetate (with 0.5% TEA) as the mobile phase. R_f(TLC) = 0.1. Yield: 287 mg (60%). ^{1}H NMR (ppm in $CDCl_3$): δ 8.51 (d, J = 2.2 Hz, 1H), 8.49 (d, J = 2.2 Hz, 1H), 8.18 (s, 1H), 8.10 (s, 1H), 7.60 (br d, 1H), 7.57 (d, J = 2.2 Hz, 1H), 7.26 (m, 8H), 6.73 (m, 2H), 4.25 (dd, J = 8.8 Hz, J = 4.4 Hz, 1H), 3.31 (m, 2H), 2.43 (s, 3H), 1.45 (s, 9H). $[\alpha]_D^{26}$ = +2.9 (c = 0.24 g/mL, in MeOH).

Preparation of (*N*-*tert*-Butoxycarbonyl-2-amino-3-(4′-methyl-2,2′-bipyridin-4-yl)propanoic acid)bis(2,2′-bipyridine)ruthenium-(II) *(2)*. All manipulations were carried out under conditions of low light. The protected bipyridine amino acid **1** (505 mg, 1.41 mmol) was suspended in a 70% ethanol (60 mL) solution. To this solution was added *cis*-dichlorobis(2,2′-bipyridine)ruthenium(II) (735 mg, 1.41 mmol). The reaction mixture was refluxed for 8 h. During the reaction, the color changed from dark purple to dark orange. After the reaction, the mixture was cooled to room temperature. The solvent was removed by rotary evaporation, and the residue was set aside overnight. The compound was purified by column chromatography using CM-sepharose resin (Pharmacia, LKB), with an ammonium chloride step gradient. A light yellow band was removed with 0.01 M NH_4Cl, a broad, orange band was eluted with 0.1 M NH_4Cl, and a red-orange band was eluted with 1 M NH_4Cl. A purple band remained on the column, most likely unreacted dichlorobis(2,2′-bipyridine)ruthenium(II). The desired product eluted at 0.1 M NH_4Cl and was precipitated out of solution by addition of saturated ammonium hexafluorophosphate. The precipitate was filtered and washed several times with water and ether. A hard, crusty orange residue remained. Yield: 70% (1.09 g, 0.987 mmol). Mp: >230 °C dec. UV (CH_3CN): λ, nm (ϵ, M^{-1} cm^{-1}), 245, 285 (49000), 324(sh), 356, 430, 455 (14200). Electrospray and MALDI-TOF MS (m/z): calcd for $C_{39}H_{39}N_7O_4P_2F_{12}Ru$ 770.54 [$(M - 2PF_6)^+$], found 770 [$(M - PF_6)^+$], 916.2. ^{1}H NMR (ppm in CD_3CN): δ 8.50 (m, 5H), 8.38 (d, J = 5.6 Hz, 1H), 8.04 (m, 4H), 7.78 (d, J = 5.2 Hz, 1H), 7.71 (m, 2H), 7.62 (m,

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2H), 7.56 (m, 1H), 7.40 (m, 4H), 7.26 (dd, J = 5.2, 1.2 Hz, 2H), 5.70 (td, J = 9.8, 1.2 Hz, 1H), 4.47 (qd, J = 9.6, 3.2 Hz, 1H), 3.39 (dd, J = 14.8, 3.8 Hz, 1H), 3.04 (td, J = 10.2, 3.1 Hz, 1H), 2.53 (s, 3H), 1.23 (d, 9H). Anal. Calcd for $C_{39}H_{39}N_7O_4P_2F_{12}Ru_1$: C, 44.17; H, 3.68; N, 9.25. Found: C, 44.03; H, 3.96; N, 9.30.

Solid-Phase Peptide Synthesis (SPPS). The peptide Ac-AKAAAAKAAAABAAAAHAAAHA-$CONH_2$ (Ac = acetyl, A = alanine, K = lysine, H = histidine, B = ruthenium amino acid) was synthesized using BOC/benzyl chemistry.[13] The couplings were carried out in DMF, with BOP as the coupling agent. HATU was used for recouplings, when necessary. A *p*-MBHA resin was used to provide a carboxyamide unit at the C-terminus after cleavage of the peptide from the resin. Due to the bulkiness of the side chain of the ruthenium amino acid, a different coupling scheme was employed for this amino acid.[14] The resin was transferred to a vial and suspended in dry 25% DMSO in toluene. A 2-fold excess of the ruthenium amino acid and of the HATU was added to the vial. The reaction was heated to 50 °C in a water bath (the vial was sealed tightly) and run for 24 h. The ruthenium amino acid was recoupled to ensure complete coupling. After the coupling of the ruthenium amino acid, any unreacted peptide was capped by acetylation. Kaiser tests[13] were performed after each coupling to verify completion. The final peptide was cleaved from the resin with HF. After removal from the resin, the crude product was run down a CM-sepharose column with an ammonium chloride gradient, to convert any hexafluorophosphate counterions to chloride, thereby increasing water solubility for HPLC purification. The crude product was purified by HPLC, using a reversed-phase C18 semipreparatory scale column (Vydac, model 218TP1022) and a water/acetonitrile gradient containing 0.1% TFA. The purity was analyzed with an analytical column. The purified peptide was also characterized by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Electrospray and MALDI-TOF MS (*m*/*z*): calcd for Ac-AKAAAAKAAAABAAAA-HAAAHA-$CONH_2$ 2450.02 $[M - 2Cl]^+$, found 2453. UV (H_2O): λ, nm, 245, 285, 324(sh), 356, 430, 455. Amino Acid Anal. Calcd for Ac-AKAAAAKAAAABAAAAHAAAHA-$CONH_2$: Ala, 17; His, 2; Lys, 2; Rubpy, 1. Found: Ala, 17.06; His, 2.05; Lys, 1.94; Rubpy, 0.88.

Fluorescence Spectroscopy and Emission Lifetime Measurements. Fluorescence spectra were obtained on a Spex Fluorolog II spectrofluorimeter. Emission and excitation spectra were obtained for the BOC-protected ruthenium amino acid in water, 25% TFE in water, and acetonitrile and for the peptide in an aqueous buffer (1 M NaCl, 1 mM sodium phosphate, 1 mM sodium borate, 1 mM sodium citrate, pH 7). For the emission spectra, the compounds were excited at 450 nm and the emission was scanned from 500 to 800 nm. For the excitation spectra, the compounds were scanned from 300 to 600 nm and the emission wavelength was set at 635 nm, the maximum intensity found in the emission spectrum.

The emission lifetimes of the amino acid and the peptide were measured using a nitrogen/dye laser tuned to 450 nm. The emission from the sample was measured by a photon multiplier tube (PMT) set perpendicular to the sample, after the light was passed through a monochromator set at 650 nm. The signal from the PMT passed through a LaCroy 8013A A/D converter, set at 5 ns per data point. The software for obtaining the data was Waveform Catalyst (LeCroy).

The amino acid was dissolved in water, D_2O, acetonitrile, or either water or D_2O with 20% TFE. The peptide was dissolved in a buffer (100 mM NaCl, 20 mM sodium phosphate, adjusted to pH 7) in either water or D_2O. Solutions (20%) of TFE were also used. The concentration ranges were between 5 and 15 μM. The sample was degassed in a quartz cell by bubbling argon through the solution for 30 min to remove O_2, and then the cell was sealed. Twenty-five laser pulses were averaged for each measurement. The data were analyzed with SigmaPlot (version 4.0, SSPS, Inc.) and fit to a single-exponential decay. A temperature study was also performed on the ruthenium amino acid, ranging from 0 to 60 °C, in water. The sample cell was temperature-controlled with a thermostated cell holder (Hitachi), and the temperature was monitored with a Digisense thermocouple (Cole-Palmer). The emission decay data were fit to a single-exponential decay. The inverse of the lifetime, $1/\tau = k_{obs}$, was then plotted versus temperature to extract the values for activation energy, E_a, k_o, and $k_r + k_{nr}$ according to eq 1.[15]

$$k_{obsd} = (k_r + k_{nr}) + k_o \exp(-E_a/RT) \quad (1)$$

Circular Dichroism (CD) Measurements. Circular dichroism measurements were done with a Jasco J-500C spectropolarimeter. The CD spectrometer was calibrated with (1*S*)-(+)-10-camphorsulfonic acid.[16] CD spectra were scanned from 350 to 200 nm. The peptide concentrations ranged from 5 to 10 μM. The peptide was dissolved in 1 M NaCl, 1 mM sodium borate, 1 mM sodium phosphate, and 1 mM sodium citrate at pH 7. The temperature of the sample cell was maintained at or near 0 °C with a circulating water bath.

TFE titrations were performed on the peptide in 5% increments until the ellipticity leveled off. The temperature dependence of ellipticity was also measured from 0 to 60 °C. Both experiments were monitored at 222 nm. The ellipticity values were converted to fractional helicity using eq 2,[17]

$$f_H = (\theta_{obsd} - \theta_C)/(\theta_H - \theta_C) \quad (2)$$

where θ_{obsd} is the measured ellipticity, θ_H is the baseline ellipticity for a complete helix, and θ_C is the baseline ellipticity for a random coil. The values for θ_H and θ_C as a function of temperature, T, are[17]

$$\theta_H = (-44000 + 250T)(1 - 3/N_r) \quad (3)$$

$$\theta_C = 2220 - 53T \quad (4)$$

where N_r is the number of amino acid residues in the peptide. The value of −44000 is the per residue ellipticity for an infinite helix, 2220 is the per residue value for a random coil at 0 °C in H_2O, and 3 is the number of non-hydrogen-bonded carbonyl units at the C-terminus.[17] For f_H values in TFE, θ_C values as a function of [TFE] at 0 °C were taken from ref 18.

The partition function, Z, for the Lifson−Roig helix−coil model was calculated as described in ref 17 using the statistical weight matrix, **M**, given in ref 17. The helix propagation parameter, w, for each naturally occurring amino acid in the 22 amino acid peptide is taken from ref 17 as is the N-cap, n, statistical weight for the coil residue next to the N-terminus of a helical segment. The helix

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initiation parameter, v, is taken as $v^2 = 0.0013$.[17] These parameters have been evaluated experimentally from a large database of alanine-based peptides similar to the peptide used here.[17] The fractional helicity at each residue, $f_H(i)$, along the helix is given by eq 5,[19]

$$f_H(i) = (\delta \ln Z)/(\delta \ln w_i) \quad (5)$$

where i is the sequence position of the residue. The overall calculated fractional helicity, f_H(calcd), is given by eq 6, where $N = N_r - 2$, or 20 in our case since the end residues of a peptide cannot be constrained to helical ϕ, ψ angles.

$$f_H(\text{calcd}) = \sum_n^i f_H(i)/N \quad (6)$$

The only unknown in evaluation of f_H(calcd) for the peptide studied here is the helix propagation parameter, w, for the unnatural metalloamino acid. This parameter is adjusted until f_H(calcd) in eq 6 equals f_H evaluated from circular dichroism data with eq 2. The Lifson−Riog partition function, Z, and eqs 5 and 6 were evaluated using the program Helix2[17,20] or with a locally written MathCad Professional, v. 7.03 (MathSoft, Inc.), file.

The temperature data were fit to the Lifson−Roig helix−coil theory, using a homopolymer model,[18] as given by eq 7, where T_o

$$\ln \langle w \rangle = \ln \langle w_o \rangle - (\Delta H/RT)(1/T - 1/T_o) \quad (7)$$

$= 273.15$ K, $\langle w \rangle$ is the mean helix propagation parameter for the homopolymer at temperature T, and $\langle w_o \rangle$ is the mean helix propagation parameter at temperature T_o. The program Helix2[17,20] was used to obtain the best helical propagation parameter $\langle w \rangle$ at each temperature such that f_H(calcd) matched the measured fractional helicity (eq 2). A plot was made of $\ln \langle w \rangle$ versus $1/T - 1/T_o$, and $\ln \langle w_o \rangle$ and ΔH values were extracted from the intercept and slope, respectively.

Kinetic Hydrogen−Deuterium Exchange NMR Measurements. The peptide was dissolved in an aqueous buffer containing 100 mM NaCl and 20 mM sodium phosphate (pH adjusted to 5.5), at a concentration of approximately 2 mM. The sample was lyophilized to dryness. The solution used for the exchange experiment consisted of 5 mM acetic acid-d_4 in D_2O, adjusted to a pD of 3.5. The buffer, sample, and NMR tube spinner were kept on ice. The NMR probe was set at 1.0 °C. After addition of the buffer, the first NMR spectral acquisition was typically obtained within 5 min. Multiple one-dimensional spectra were obtained, with the methyl group on the bipyridine amino acid used as an integration reference against the entire aromatic and amide region. Data were acquired with a Varian Inova 500 MHz NMR spectrometer at the NMR Center of the University of Colorado Health Sciences Center. The spectral width was 6000 Hz, and the data were collected with an optimized 90° pulse. The FID was a sum of 32 scans with 4096 points and an acquisition time of 0.34 s. During the first 30 min, spectra were collected every minute. Then spectra were collected every 4 min for 5 h and last every 15 min for 15 h. The pH* was measured at the end of each of the two experiments carried out, giving pD values of 3.47 and 3.92, respectively.

The integrated intensity data for the aromatic and amide region were converted to fractional proton occupancy. A linear extrapolation of the integrated intensity between $t = 5$ min and $t = 15$ min after the addition of D_2O to $t = 0$ min was used to estimate 100% amide proton occupancy. The final integrated intensity was assumed to represent 0% amide proton occupancy. The C-terminal carboxyamide and the lysine amines are expected to exchange immediately upon dissolving in D_2O and thus should not interfere with the measurement of amide exchange kinetics.[9] Proton occupancy was plotted versus time. The curves were fit to the Lifson−Roig helix−coil theory, assuming that a proton cannot exchange when it is in a hydrogen bond.[9] The fractional proton occupancy as a function of time, $f_o(t)$, can be expressed as in eq 8,[9] where N_r

$$f_o(t) = \sum (1/N_r) \exp[-(k_{int}[1 - f_B(i)])t] \quad (8)$$

is the number of residues in the peptide, k_{int} is the exchange rate constant for a residue with a non-hydrogen-bonded amide NH, $f_B(i)$ is the probability that an amide NH is hydrogen-bonded, and t is time. The exchange rate constant, k_{int}, at the pD of each experiment was calculated using the program Sphere,[21] and the experimental parameters of Bai et al.[22] The probability factor, $f_B(i)$, was obtained using the program Helix2,[17,20] such that $f_B(i) = f_H(i - 2)$, where $f_H(i - 2)$ is the probability that the residue at position $i - 2$ has helical ϕ, ψ angles (in the Lifson−Roig helix−coil theory, helical ϕ, ψ angles at residue $i - 2$ are directly related to a hydrogen bond between the carbonyl at residue $i - 4$ and the amide NH of residue i). The experimentally determined helix propagation parameters for the naturally occurring amino acids[17] in the peptide were used in the calculation of $f_B(i)$ as described above (eq 5) using the program Helix2.[17,20] The helix propensity factor, w, was varied for the ruthenium amino acid in the calculation of the $f_B(i)$ values at each site so as to minimize the value of $\sum(\Delta \text{ fractional occupancy})^2$ for eq 8 relative to the experimental data.

Results and Discussion

Improved Synthesis of the Bipyridine Amino Acid. The main improvement in the synthesis of the bipyridine amino acid **1**[7] involves the use a novel chiral phase-transfer catalyst developed by Corey et al.[12] In the previously reported synthesis,[7] we used the commercially available chiral phase-transfer catalyst (8*S*,9*R*)-(−)-*N*-benzylcinchonidinium chloride in the reaction of 4-(bromomethyl)-4′-methyl-2,2′-bipyridine **(3)** with *N*-(diphenylmethylene)glycine *tert*-butyl ester **(4)**. The ee of the L isomer of 2-amino-3-(4′-methyl-2,2′-bipyridin-4-yl)propanoic acid **(6)** was 66%. Enzymatic resolution of the D and L isomers added two additional steps to the synthesis. Use of the novel phase-transfer catalyst *O*(9)-allyl-*N*-9-anthracenylmethylcinchonidinium bromide,[12] as outlined in Scheme 1, improves the ee of amino acid **6** to >99%. The initially produced *N*-(diphenylmethylene)-2-amino-3-(4′-methyl-2,2′-bipyridin-4-yl)propanoic acid *tert*-butyl ester **(5)** was hydrolyzed in 6 N HCl to produce the free amino acid, as previously described,[7] and then analyzed on a CrownPak(+) chiral column. Only a single peak was observed in the chromatogram, with a retention time of 11.69 min. This retention time corresponds to that observed for the L isomer in the mixture of L and D isomers of amino acid **6**, produced with the previously used phase-transfer catalyst (retention times of 11.69 and 9.78 min, respectively).

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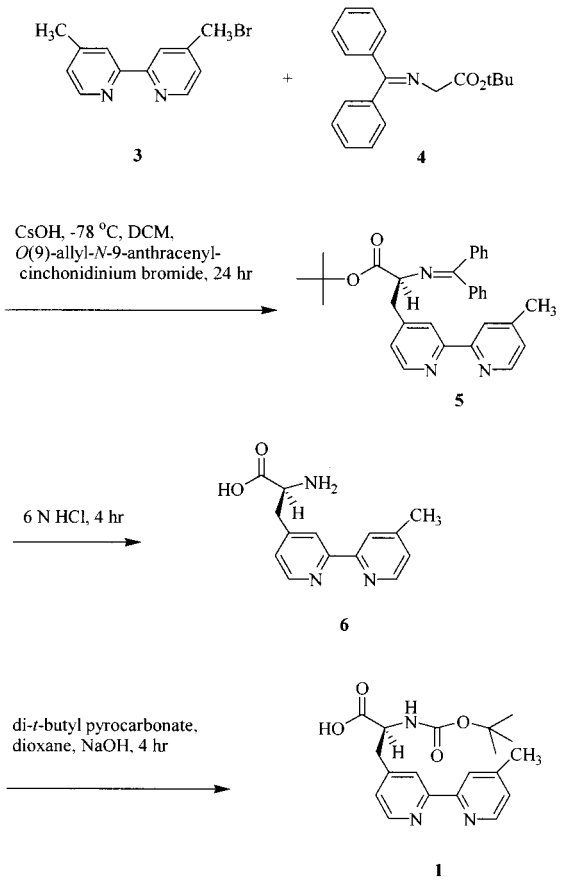
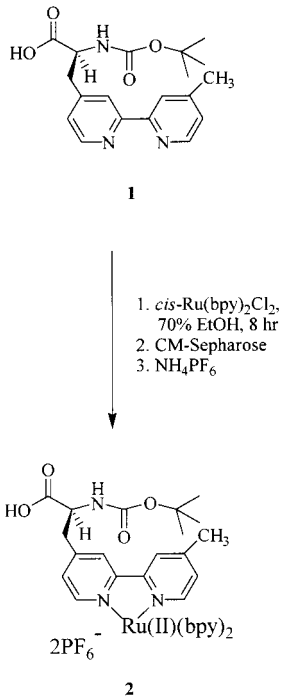
Scheme 1



Scheme 2



The BOC-protected form of the amino acid (**1**) used in the synthesis of the metalloamino acid was prepared as described previously.[7]

Metalloamino Acid. The metalloamino acid **2** was produced in one step (Scheme 2) by reaction of the BOC-protected amino acid **1** with *cis*-dichlorobis(2,2′-bipyridine)-ruthenium(II). Reaction with the unprotected amino acid was unsuccessful, presumably due to interference from the α-amino group. Purification by ion-exchange chromatography was followed by precipitation with saturated ammonium hexafluorophosphate, yielding a homogeneous product. The NMR spectrum reveals a splitting of the BOC peak, indicating that the cis and trans isomers about the CN bond of the BOC urethane functionality are of similar stability, not unlike X−Pro peptide bonds.[23] The UV/vis spectrum (see the Experimental Section; see also the Supporting Information, Figure S1) is similar to the spectrum for tris(2,2′-bipyridine)ruthenium(II).[24]

The mass spectrum of the metalloamino acid was obtained using both MALDI-TOF and electrospray methods. Both revealed a peak at *m*/*e* 771, which corresponds to the amino acid with no counterions, and a peak at *m*/*e* 917, where one hexafluorophosphate ion remains associated with the metalloamino acid. The electrochemical potential, $E_{1/2}$, for the metalloamino acid was found to be 1.22 ± 0.05 V vs SCE in acetonitrile. The shift in the potential was very small (40 mV decrease) when water was added to the solution to a ratio of 1:1 H_2O/CH_3CN. The potential is similar to those of $Ru^{II}(bpy)_3$ ($E_{1/2}$ = 1.28 V vs SCE) and $Ru^{II}(DMB)_3$ ($E_{1/2}$ = 1.15 V vs SCE) measured under the same conditions.[25]

Table 1. Emission Lifetime Data for the Ruthenium Amino Acid **2** and the Metallopeptide[a]

solvent	amino acid **2**	metallopeptide[b]	lit. value[c]
D_2O	1178 ± 5	1142 ± 15	985
H_2O	631 ± 3	644 ± 11	635
D_2O and TFE	1286 ± 17	1302 ± 4	
H_2O and TFE	997 ± 6	863 ± 11	
acetonitrile	994 ± 4		870
H_2O, 58.5 °C	276 ± 1	305 ± 4	300[d]

[a] All lifetimes in nanoseconds and at 0 °C, unless otherwise noted. [b] With 100 mM NaCl, 20 mM sodium phosphate, adjusted to pH 7. [c] Data are for $Ru(II)bpy_3$ from Table 6.3 in ref 24 unless otherwise noted. [d] Data are for $Ru(II)bpy_3$ from ref 15a.

When steady-state fluorescence measurements were performed in water, the emission spectrum revealed a broad peak with maximal intensity at ∼635 nm. The excitation spectrum showed a peak at 467 nm, with a shoulder at around 440 nm. Both excitation and emission spectra are similar to the spectra for $Ru^{II}(bpy)_3$.[24]

The emission lifetime of the BOC-protected metalloamino acid was measured in several solvents (Table 1). The data, in all cases, fit well to a single-exponential decay. The emission lifetimes are similar to $Ru^{II}(bpy)_3$,[24] indicating that the photophysical properties are not strongly perturbed by substitution of the bipyridine amino acid for one of the bipyridine ligands.

The temperature dependence of the emission lifetime of the amino acid was monitored from 0 to 60 °C in water.

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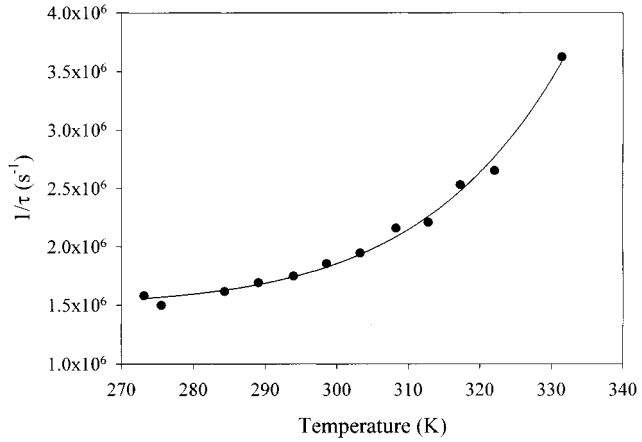
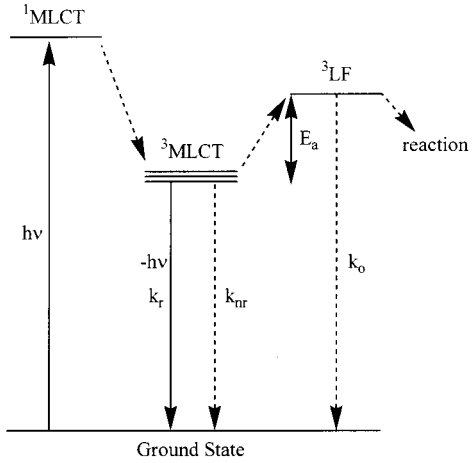


Figure 1. Temperature dependence of the emission lifetime for the metalloamino acid in water. The filled circles are the data, and the solid line is a fit of the data to eq 1. The concentration was 14.9 μM. The excitation wavelength was 450 nm. The emission wavelength was 650 nm.

Scheme 3



The lifetime decreases as the temperature increases (Figure 1). This indicates that decay from the excited state is an activated process.[15] The model typically used to describe this behavior is shown in Scheme 3.[15] The data were fit to eq 1 (Experimental Section) to obtain E_a, k_o, and $k_{nr} + k_r$. The activation energy was 3900 $\pm$ 300 cm^{-1}, which compares well with 3600 cm^{-1} for $Ru^{II}(bpy)_3$.[15a] The magnitudes of k_o and $k_{nr} + k_r$ were found to be (2 $\pm$ 1) $\times$ 10^{13} and (1.50 $\pm$ 0.04) $\times$ 10^6 s^{-1}, respectively, also typical for $Ru^{II}(bpy)_3$ complexes.[15,26]

Metallopeptide. The metalloamino acid was incorporated into a structure-forming peptide: Ac-AKAAAAKAAAA-BAAAAHAAAHA-NH_2. The sequence of this peptide was based on a set of peptides designed by Baldwin and co-workers.[8] The high alanine content of this peptide enhances α-helix formation, and the lysines and histidines are inserted to achieve water solubility and are spaced along the helix so as to prevent aggregation.

The steric bulk of the ruthenium amino acid required that more vigorous methods be used to achieve efficient coupling to the peptide chain. DMSO/toluene, 1:3, was used as solvent to achieve greater swelling of the resin and to allow the reaction to be run at higher temperature.[14] Adequate coupling yields (negative Kaiser test) were achieved by this method.

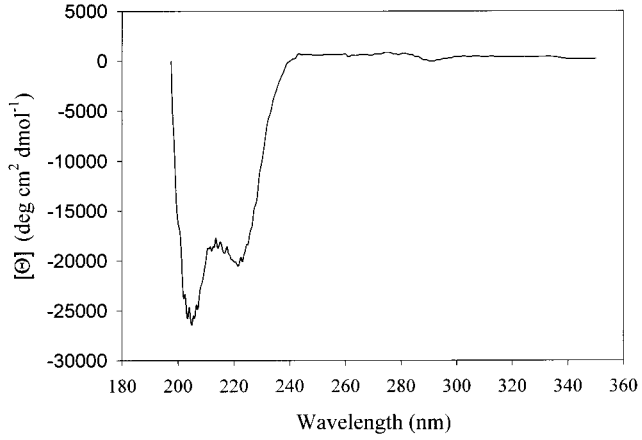


Figure 2. CD wavelength scan of the metallopeptide. The temperature was −0.6 °C. The concentration of the sample was 9.44 μM. The buffer was 1 M NaCl, 1 mM sodium phosphate, 1 mM sodium citrate, 1 mM sodium borate, pH 7. The ellipticity is reported as mean molar residue ellipticity, $[\theta] = (M_r\psi/100lc)/N_r$, where M_r is the molecular weight of the sample, ψ is the measured ellipticity of the sample in degrees, l in the path length of the cell in decimeters, c is concentration in grams per milliliter, and N_r is the number of residues in the peptide.

The peptide was characterized by mass spectrometry after purification. The MALDI-TOF and electrospray mass spectra gave the expected molecular ion peak within error of the expected value of *m*/*e* 2450. The UV/vis spectrum and the steady-state fluorescence spectrum were similar to the corresponding spectra for the metalloamino acid (Figures S1−S4 in the Supporting Information), indicating that the peptide structure does not significantly affect the physical properties of the metal complex. The amino acid analysis reveals the expected amino acid content for the peptide (see the Experimental Section).

CD was used to characterize the structure of the peptide (Figure 2). There are minima at 222 and 209 nm, indicative of an α-helix. At 290 nm, there is a subtle deflection, indicating a slight excess of the Δ isomer[27] of the ruthenium complex in the peptide. A TFE titration was performed on the peptide to obtain a helix induction curve (Figure 3). The profile of this curve is similar to curves of other polyalanine peptides, where the ellipticity levels off at higher TFE concentrations.[18]

The fractional helicity at 0 °C derived from the mean molar residue ellipticity using eqs 2−4 in the Experimental Section is 0.67 $\pm$ 0.04. The program Helix2[17,20] was used to assess the helix propagation parameter, *w*, for the ruthenium amino acid. The reported values of *w* for the other amino acids (His, 0.36; Lys, 1.0; Ala, 1.7)[17] in the peptide were used by Helix2 to calculate the partition function and f_H(calcd) for the peptide (eq 6). The value of *w* for the ruthenium amino acid was varied until f_H(calcd) matched the observed helicity, f_H (eq 2), for the peptide. A helix propagation parameter $w = 0.42 \pm 0.13$ was obtained with this procedure. Since the

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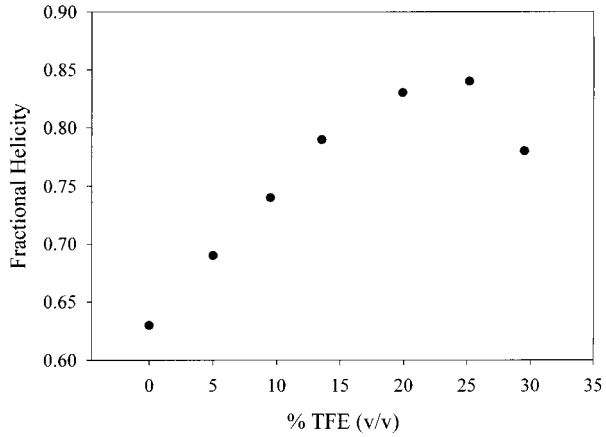

Figure 3. TFE induction curve for the metallopeptide. The temperature was 0.5 °C. The concentration was 6.39 μM. The buffer was 1 M NaCl, 1 mM sodium citrate, 1 mM sodium phosphate, 1 mM sodium borate, adjusted to pH 7. The 2,2,2-trifluorethanol concentration was increased in 5% increments. Ellipticity measurements were made at 222 nm and converted into fractional helicity as described in the Experimental Section.

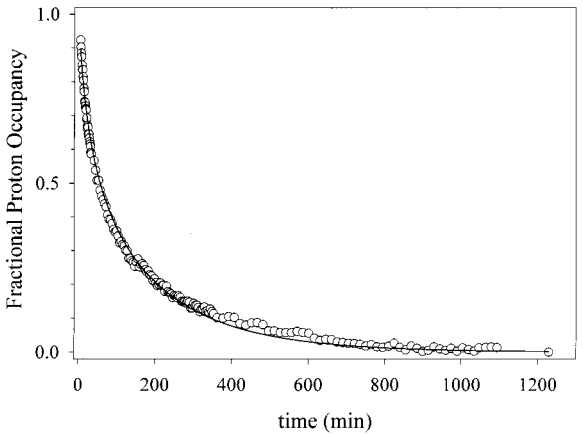

Figure 4. Kinetic amide hydrogen−deuterium exchange plot for the metallopeptide. The percent proton occupancy (open circles) as a function of time was determined as described in the Experimental Section. The temperature of the NMR probe was 1 °C. The pD of the solution was measured after the experiment and was 3.47. The metallopeptide concentration was 2 mM. The solid curve is the best fit to eq 8 in the Experimental Section obtained by varying the helix propagation parameter, *w*, for the metalloamino acid.

helix propagation parameters for the naturally occurring amino acids were obtained using a large database of alanine-based peptides[17] containing substitutions of each of the naturally occurring amino acids, we are effectively obtaining *w* for the ruthenium amino acid relative to a large database of similar peptides. The aromatic character of the ruthenium amino acid is expected to have an effect on the far-UV region of the CD spectrum.[28] However, the effect is likely to be small, since the CD spectrum of the peptide indicates that the ruthenium amino acid in the peptide exists as a nearly racemic mixture of the Δ and Λ isomers.

To be certain that the CD estimate of *w* for the ruthenium amino acid is not strongly perturbed by the effect of the aromatic character of the ruthenium amino acid, a kinetic hydrogen−deuterium exchange experiment observed by NMR was conducted. The data were fit to the Lifson−Roig helix−coil theory, as described in the Experimental Section (Figure 4). As with the fit to the CD data, the propagation parameters for the other amino acids in the peptide are known from studies on a large database of alanine-based peptides. Thus, the only adjustable parameter in the fit of the experimental data to the Lifson−Roig helix−coil theory using eq 8 (Experimental Section) is the helix propagation parameter, *w*, for the ruthenium amino acid. A value of $w = 0.57$ was obtained at both pD values used for this experiment. Thus, interference in the intrinsic peptide region of the CD spectrum, due to the ruthenium amino acid, appears to be minimal.

From the CD and NMR data, it can be seen that the peptide has a considerable amount of helical content. The profile of the wavelength scan from the CD data indicates strong α-helix formation. The CD and NMR data are consistent with a helical propagation factor, *w*, of 0.5 ± 0.1 at 0 °C. This value is similar to those of amino acids such as methionine, glutamate, and glutamine,[17] which have moderate helix propensities, and is consistent with the high helical content of this peptide. In previous work,[7] we have studied the structural properties of a peptide with the same sequence as the one studied here, except that the bipyridyl amino acid **6** (see Scheme 1) is at the sequence position of the ruthenium amino acid in the peptide studied here. Using data obtained at 25 °C for this peptide,[7] the experimental fractional helicity, f_H, obtained from CD data yields a value of $w = 1.0 \pm 0.3$ at 0 °C using the Lifson−Roig helix−coil theory to evaluate f_H(calcd) (eq 6, Experimental Section). Thus, conversion of the bipyridyl amino acid **6** to a ruthenium(II) tris(bipyridyl) amino acid reduces the helix propensity. This observation is in line with the general observation that increasing steric bulk for the side chain of an amino acid lowers its helical propensity. Of the metal-binding amino acids discussed in the Introduction, only the aminodiacetic acid side chain was incorporated into peptides expected to form α-helices. In general, the reported mean residue ellipticity of these peptides indicated good helix formation, suggesting that this amino acid has reasonable helical propensity. The data were not analyzed with the helix−coil model to obtain helix propagation parameters, so no quantitative comparison of the helix propensity of this unnatural amino acid with the ruthenium(II) tris(bipyridyl) amino acid reported here is possible.

The output of the Helix2 partition function analysis indicates that the fractional helicities of the individual amino acid residues are >0.6 between Lys 2 and Ala 16 of the metallopeptide, reaching a maximal value of 0.86 at Ala 8. The fractional occupancy of the ruthenium bipyridine amino acid (sequence position 12) in the helical conformation is 0.78. The partition function analysis predicts significant fraying of the helix at the C-terminus. The low helical propensity of histidine is the primary cause of the predicted fraying of the helix at the C-terminus. The fractional helicities of amino acids from His 17 to the end of the peptide are all less than 0.5 and become progressively smaller the closer the residue is to the C-terminus. Direct demonstration of the fraying of helix ends predicted by the Lifson−Roig helix−

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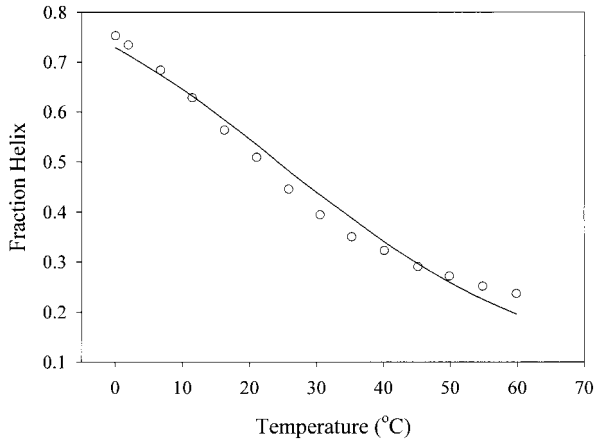

Figure 5. Temperature denaturation of the metallopeptide as measured by CD spectroscopy. The temperature was raised in 5 °C increments. The concentration was 9.44 μM. The open circles are the data, and the solid line is a fit to the Lifson−Roig helix−coil theory using a homopolymer model as described by eq 7 in the Experimental Section. The buffer was 1 M NaCl, 1 mM sodium citrate, 1 mM sodium phosphate, 1 mM sodium borate, adjusted to pH 7.

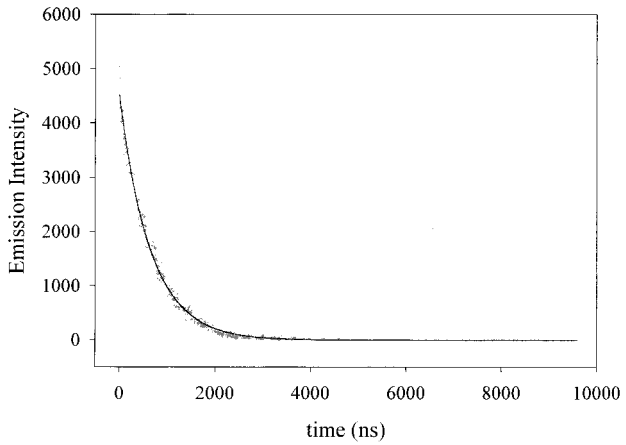

Figure 6. Emission lifetime decay of the metallopeptide in aqueous buffer at 0 °C. The concentration of the metallopeptide was 14.9 μM. The excitation wavelength was 450 nm, and the emission wavelength was 650 nm. The solid gray dots are the data, and the fit to a single-exponential decay is shown with the solid line. The lifetime for the fit is given in Table 1.

coil model has been observed directly by NMR methods using ^{15}N-labeled alanine-based peptides[29] similar to the alanine-based peptide used in this work.

The temperature dependence of the structure content of the peptide was monitored by CD spectroscopy over a range of 0−60 °C (Figure 5). The data reveal a broad sigmoidal curve, going from a substantially helical peptide at low temperatures to a near random coil at higher temperatures, similar to other helical peptides.[18,30] The data were fit to the Lifson−Roig helix−coil theory, as described in the Experimental Section. The homopolymer model used fits the data reasonably well, even though the lower helix propensities of the histidines and the ruthenium amino acid, relative to the lysines and alanines in the peptide, is expected to compromise the homopolymer approximation. From the curve fit, the values for w_o and ΔH (eq 7, Experimental Section) were extracted. The enthalpy was −677 cal/mol, which is lower than the reported value of about −1000 cal/mol for an alanine/lysine peptide.[18,30] The lower value of ΔH observed here may result from the homopolymer approximation being a poorer approximation than for the previously studied peptides. The helical propensity at 0 °C, w_o, is 1.37, which is lower than the value of ∼1.6 found for alanine/lysine peptides.[31] This result is expected due to the histidines and the ruthenium amino acid, which have lower helical propensities than the lysine and alanine residues in the alanine/lysine peptides used in previous work.[31] The breadth of the transition is typical for a peptide of this size.

The emission lifetime of the ruthenium amino acid in the peptide was measured using a nitrogen/dye laser, with an excitation pulse set at 450 nm and emission detection set at 650 nm. The lifetimes were measured in several solvents (Table 1). The data, in all cases, fit to a single-exponential decay (Figure 6). The emission lifetime at 0 °C in aqueous buffer is similar to that of the BOC-protected ruthenium amino acid in water, indicating that the photophysical properties are not strongly perturbed by incorporating the ruthenium amino acid into a peptide. The data also show that the structural properties of the peptide have minimal impact on the emission lifetime of the ruthenium amino acid in the peptide when the helical structure is enhanced (TFE added) or destroyed (high temperature).

Conclusion

A metalloamino acid has been synthesized and characterized, and its physical properties have been examined. These studies have revealed that the metalloamino acid behaves very much like $Ru^{II}(bpy)_3$. The metalloamino acid has been successfully incorporated into a water-soluble, alanine-based peptide. The metalloamino acid is stable to the SPPS conditions that were used to prepare the peptide. Despite the steric bulk of the side chain of the metalloamino acid, it still has a moderate helix propensity, which allows its inclusion in structure-forming peptides. This metalloamino acid will now allow for facile site-directed incorporation of a metal complex into peptides with multiple binding sites for metals.

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Supporting Information Available: UV/vis spectra and emission spectra of the BOC-protected ruthenium amino acid **2** and the peptide containing the ruthenium amino acid. This information is available free of charge via the Internet at http://pubs.acs.org.

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