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Preparation and Characterization of [^{99}TcO] Apcitide: A Technetium Labeled Peptide

Lynn C. Francesconi,*,† Yongyong Zheng,† Judit Bartis,† Michael Blumenstein,† Catherine Costello,‡ and Mark A. De Rosch§

Department of Chemistry, Hunter College and the Graduate School of the City University of New York, New York, New York 10021, Mass Spectrometry Resource, Boston University Medical School, Boston, Massachusetts, and Berlex Laboratories, Inc., Montville, New Jersey

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[^{99m}TcO] apcitide (^{99m}TcO(P246)), the technetium complex of the 13 amino acid, apcitide, *cyclo*-(D-Tyr-Apc-Gly-Asp-Cys)-Gly-Gly-Cys(Acm)-Gly-Cys(Acm)-Gly-Gly-Cys-NH_2, where Apc is L-[*S*-(3-aminopropyl)]cysteine (an arginine mimetic) and Acm is the acetamidomethyl protecting group, has high affinity and selectivity for the GPIIb/IIIa receptor that is expressed on the membrane surface of activated platelets and plays an integral role in platelet aggregation and thrombus formation. Bibapcitide, a 26 amino acid, bis-succinimidomethyl ether-linked dimer of the peptide apcitide has been formulated as a single-vial, lyophilized kit having the trade name AcuTect. When sterile, nonpyrogenic sodium pertechnetate ($^{99m}TcO_4^-$) in 0.9% sodium chloride is added to the AcuTect radiopharmaceutical kit and the resulting kit is heated, [^{99m}TcO] apcitide forms. This is the first radiopharmaceutical to target acute deep vein thrombosis (DVT) in the lower extremities. We report here the preparation, purification, and isolation of the ^{99}Tc complex of apcitide and its characterization to determine the mode of binding of Tc to apcitide. [^{99}TcO] apcitide was prepared, on the macroscopic level, by reaction of $[^{99}TcOCl_4]^-$ with apcitide, purified by preparative HPLC and isolated as a trifluoroacetate salt. [^{99}TcO] apcitide can also be formed from the reaction of bibapcitide and $^{99}TcO_4^-$ in the presence of Sn(II) and glucoheptonate at 80 °C, conditions that mimic the radiopharmaceutical kit preparation. FTIR data show a Tc═O stretch at 961.2 cm^{-1}, in the range observed for anionic $[Tc^VO]^{3+}$ amide thiolate complexes. The mass spectral data is in agreement with the formula, $[C_{51}H_{73}O_{20}N_{17}S_5Tc]^-$, consistent with retention of Acm groups and the Tc binding in the Gly^{11}-Gly^{12}-Cys^{13} region of the peptide. Despite significant spectral overlap due to numerous similar amino acids, all protons of apcitide and [^{99}TcO] apcitide were unambiguously assigned. The observation of two nonequivalent Acm groups and the observation of only 10 NH–CH cross-peaks in the TOCSY and COSY spectra of [^{99}TcO] apcitide (NH–CH cross-peaks were absent for Gly^{11}-Gly^{12}-Cys^{13}), compared to all 13 cross-peaks found in apcitide, provided compelling evidence to support the ^{99}Tc binding to the terminal Gly^{11}-Gly^{12}-Cys^{13} region of apcitide.

Introduction

Deep vein thrombosis (DVT) and pulmonary embolism (PE) constitute a major health risk. Thrombus formation is the cause of occlusions and emboli in arterial and venous circulation. Approximately 70% of PE cases arise from DVT of the lower extremities.[1] Therefore early and accurate detection of DVT is important to allow prompt treatment to avoid formation of an embolus. Existing diagnostic modalities are inadequate for detecting the location and age of venous and arterial thrombus formation.[2,3]

The glycoprotein IIb/IIIa receptor (GPIIb/IIIa) is expressed on the membrane surface of activated platelets and plays an integral role in platelet aggregation and thrombus formation.[4–7] Molecules, such as fibrinogen, which mediate cell adhesion

* Author to whom correspondence should be addressed. E-mail: lfrances@hunter.cuny.edu.

† Hunter College and the Graduate School of the City University of New York.

‡ Boston University Medical School.

§ Berlex Laboratories, Inc. Current address: Vertex Pharmaceuticals Inc., Cambridge, MA.

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bind to the GPIIb/IIIa receptor via the tripeptide sequence Arg-Gly-Asp (RGD). Cyclic peptides containing the RGD (Arg-Gly-Asp) sequence have been shown to be high-affinity antagonists for the GPIIb/IIIa receptor.[4]

One approach to detection of thrombosis is to "tag" GPIIb/IIIa receptor antagonists with a radionuclide as the radiolabeled molecule should only be bound to platelets involved in the forming thrombi. Scintigraphic techniques can then be used to determine the location and extent of the active (acute) thrombi. The radionuclide of choice for diagnostic nuclear medicine is ^{99m}Tc because of its ideal physical properties (6.02 h half-life, 140 keV, γ-emission), high purity, commercial availability, and low cost.

Radiolabeled fibrinogen, fibrin, or platelets have demonstrated slow blood clearance, resulting in poor target-to-background ratios over the course of several hours after injection.[8] Fab′ fragments derived from monoclonal antibodies raised against fibrin, labeled with ^{99m}Tc also cleared the blood pool too slowly to provide reliable diagnosis.[9]

Another strategy involving labeling small peptide sequences incorporating the RGD sequence, or mimetics of this tripeptide sequence, appears to be promising. For example, "bifunctional" chelating agents based on the N_2S_2 ligand allow the formation of the $TcO(N_2S_2)$ unit derivatized with a pendant carboxylate group; the carboxylate group can be further derivatized with a small peptide via the active ester.[10,11] Another bifunctional chelating approach uses the hydrazidonicotinamide-conjugated GPIIb/IIIa antagonist (HYNICtide) moiety to bond to ^{99m}Tc with co-ligands, EDDA or tricine and a water soluble phosphine.[11−13] Mixed ligand experiments have been used to determine composition of [^{99m}Tc(HYNICtide)(TPPTS)(tricine)] complexes.[13]

Alternatively, a technetium-binding site can be engineered into the small peptide via judicious choice of amino acids.[14] Small peptide and peptide analogues, especially those containing thiolate sulfur donor atoms, complex with $[TcO]^{3+}$ to form small, stable structures.[15] The peptide dimethylglycyl-L-seryl-L-cysteinylglycinamide (RP294) forms stable Tc complexes[16] and has been used to tag ^{99m}Tc to a Tuftsin receptor agonist peptide sequence, threonyl-L-lysyl-L-prolyl-L-prolyl-L-arginine (TKPPR).[17] NMR characterization of the Tuftsin analogue confirms the Tc binding to the RP294 tripeptide sequence.

The thirteen amino acid peptide, apcitide (P246), has the amino acid sequence, *cyclo*-(D-Tyr-Apc-Gly-Asp-Cys)-Gly-Gly-Cys(Acm)-Gly-Cys(Acm)-Gly-Gly-Cys-NH_2 where Apc is L-[*S*-(3-aminopropyl)]cysteine (an arginine mimetic) and Acm is the acetamidomethyl protecting group. Apcitide is shown in Figure 1a; the amino acids are numbered to facilitate discussion of the data. Apcitide contains a GPIIb/IIIa receptor avid sequence (Apc-Gly-Asp) in the cyclic portion of the peptide. The peptide bibapcitide, Figure 1b, is a 26 amino acid, bis-succinimidomethyl ether-linked dimer of the peptide apcitide. Bibapcitide has been formulated as a single-vial, lyophilized kit having the trade name AcuTect. When radiolabeled with ^{99m}Tc, bibapcitide forms the radiopharmaceutical [^{99m}TcO] apcitide (^{99m}TcO(P246)), Figure 1c. [^{99m}TcO] apcitide has high affinity and selectivity for the GPIIb/IIIa receptor and binds preferentially to activated platelets. Specific uptake by acute venous thrombi was confirmed in a dog model.[18] Patient studies indicated that scintigraphy using [^{99m}TcO] apcitide is a safe and sensitive method for diagnosing acute deep vein thrombisis.[19] In phase III clinical trials, [^{99m}TcO] apcitide compared well with contrast venography, the accepted gold standard, for detecting acute DVT.[20] AcuTect is presently on the market in the US for diagnosis of DVT.

In this study, we report the synthesis, purification, and isolation of [^{99}TcO] apcitide and its characterization by FAB-MS, IR spectroscopy, and NMR spectroscopy. The goal of this study was to determine the mode of binding of technetium to apcitide. The data provide unambiguous evidence for the binding of Tc in this 13 amino acid peptide.

Experimental Section

General Comments. ^{99}Tc is a weak β^- emitter and should be handled in a laboratory approved for low level radiation, and appropriate radiation safety procedures should be followed. $NH_4{}^{99}TcO_4$ was obtained from Oak Ridge National Laboratory, Oak Ridge, TN. 30% H_2O_2 was added to an aqueous solution of $NH_4{}^{99}TcO_4$ to oxidize any $^{99}TcO_2$ present. The ammonium pertechnetate solution was standardized prior to use as previously described.[21] The reagent $[TcOCl_4]N(C_4H_9)_4$ was prepared following

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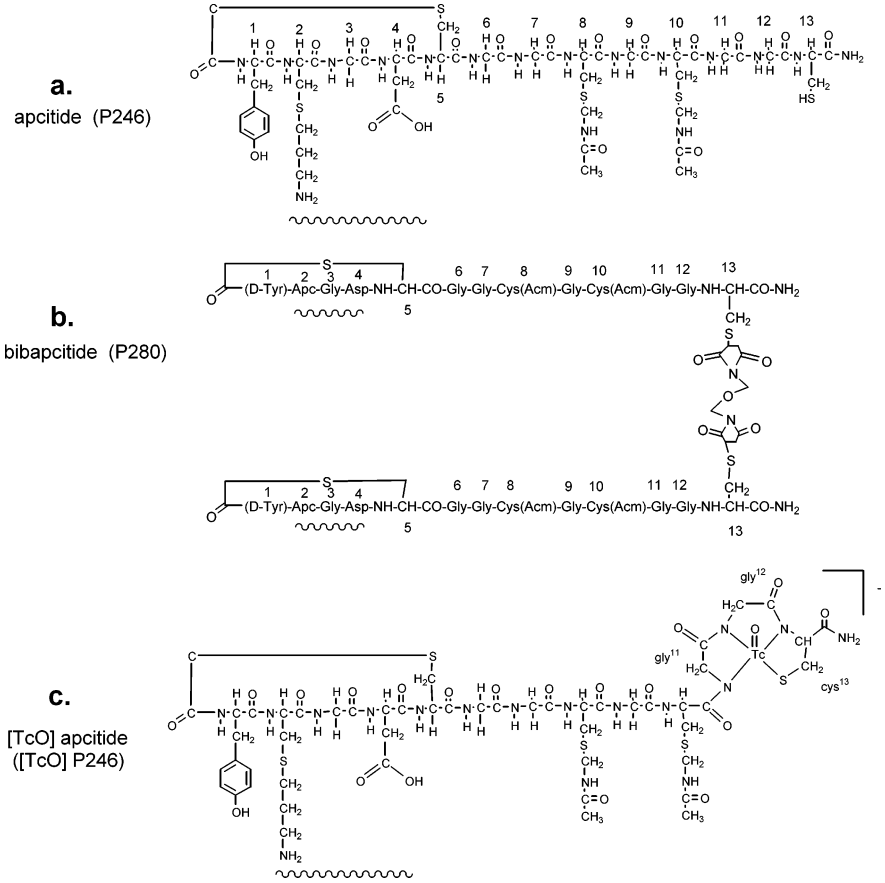

Figure 1. (a) Thirteen amino acid peptide, apcitide (P246), showing numbering system: Apc = L-[*S*-(3-aminopropyl)]cysteine, an arginine surrogate; Acm = acetamidomethyl. (b) Bibapcitide. (c) [TcO] apcitide ([TcO] P246). The receptor binding region, Apc-Gly-Asp, is indicated.

a published procedure.[22] Apcitide was synthesized and isolated as the trifluoroacetate salt, apcitide trifluoroacetate.[14] The peptide content of apcitide trifluoroacetate was 83%, and the purity was >96%. To produce the radiotracer [^{99m}TcO] apcitide, a lyophilized kit containing apcitide was reconstituted with $^{99m}TcO_4^-$ in saline and incubated at room temperature. HPLC grade acetone (Fisher), trifluoroacetic acid (Aldrich), and reagent grade DMF (Fisher) were used in this study. Water was obtained from a Millipore filtration system on the day of use. Infrared spectra were measured from KBr pellets on a Perkin-Elmer 1615 Fourier transform infrared spectrophotometer.

Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectra were obtained with a Finnigan MAT Vision 2000 instrument (Hemel Hempstead, U.K.) with a pentium PC-based data system at a 500 MHz sampling rate. The nitrogen laser (Laser Science, Inc., Newton, MA) provides 3 ns pulses at 37 nm. Spectra were recorded at near threshold irradiance. The laser shot rate was controlled manually, and selected spectra were added together. MALDI mass spectra were obtained in the negative-ion mode with accelerating voltage −5 kV. Postacceleration at the secondary electron multiplier was +18 kV. External calibration was used. The quoted error for external calibration is 0.1%, e.g. 1.5 Da for our complexes. Samples were dissolved in water (2−5 pmol/μL). The 2,5-dihydroxybenzoic acid matrix was also dissolved in water (10 g/L). The sample and matrix solutions (1 μL each) were mixed on the stainless steel target and allowed to crystallize with ambient air-drying. The target was introduced into the ion source, and the sample spots were observed with a video camera during MALDI TOF spectral analysis. For the peptide, the LSIMS spectrum was also obtained on a JEOL HX110/HX110 tandem mass spectrometer using a glycerol matrix.

A RAINEN Dynamax HPLC system, including two Dynamax model SD-200 pumps equipped with 25 mL pump heads, was used for analytical and preparative work. A Dynamax model UV-1 UV−visible detector was used to monitor UV while the γ ray of the ^{99m}Tc was monitored by a home-built detector composed of Tennelec components: power supply, high voltage supply, and amplifier in a Tennelec Minibin. The software used was the Dynamax HPLC Method Manager. For analytical HPLC, a Waters DeltaPak 15 μ C18 100 Å, 3.9 × 150 mm column was used, and for preparative work, a Waters Delta Pak 15 μ C18 300 Å, 19.0 × 300 mm was employed. The mobile phase consisted of (A) 0.1% trifluoroacetic acid (TFA) in H_2O and (B) CH_3CN/H_2O 50/50 (v/v) modified with 0.1% TFA. The gradient for analytical work was 16−24% B in 30 min, followed by 24−40% B in 10 min at a flow rate of 1 mL/min. For preparative work, the gradient was 16−24% B in 60 min, followed by 24−40% B in 20 min at a flow rate of 24 mL/min.

Collection of NMR Data. ^{1}H NMR spectra were recorded on a Varian Unity Plus 500 MHz spectrometer operating at 499.863 MHz. One-dimensional NMR experiments and two-dimensional TOCSY (total correlation spectroscopy), COSY (correlation spectroscopy), NOESY (nuclear Overhauser effect spectroscopy), and ROESY (rotating frame Overhauser effect spectroscopy) experiments were performed. Solvent suppression one- and two-dimensional experiments were accomplished using presaturation of the H_2O resonance. Two-dimensional experiments were run in the phase sensitive mode using the states method. Relaxation delays

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Table 1. Chemical Shift Data (δ, ppm) for Apcitide[a]

amino acid	NH(amide)	α-CH	other protons in AA
Tyr[1]	8.82	4.52	3.06, 2.94 (CH_2) 3.44, 3.37 ($CO-CH_2-S$) 6.85, 7.15 aromatic
Apc[2]	8.69	4.43	2.79 (CH_2) 3.06 (CH_2-NH_2) 2.47 ($-S-CH_2$) 1.89 ($CH_2-CH_2-CH_2$)
Gly[3]	8.43	3.94, 4.06	
Asp[4]	8.53	4.62	2.69[b]
Cys[5]	8.46	4.45	3.03[b]
Gly[6]	8.57	4.00	
Gly[7]	8.42	4.01	
Cys[8]	8.50	4.59	2.95, 3.13
Gly[9]	8.65	3.99	
Cys[10]	8.46	4.59	2.94, 3.11
Acm[8,10]	8.67	4.30	2.02 (CH_3)
Gly[11]	8.71	3.99	
Gly[12]	8.34	4.01	
Cys[13]	8.39	4.51	2.96
C-terminal amide	7.33, 7.79		

[a] NMR spectral data measured in in 95%/5% H_2O/D_2O, pH = 4.0, T = 7 °C. Chemical shifts are accurate to ±0.02 ppm. Since the shifts for α-CH protons are determined from the F1 dimension of the two-dimensional experiments, the uncertainty in these shifts may be slightly greater. [b] Methylene protons within 0.05 ppm are given the same chemical shift value.

Table 2. Chemical Shift Data (δ, ppm) for [^{99}TcO] Apcitide[a]

amino acid	NH(amide)	α-CH	other protons in AA
First 10 Amino Acids (Nonbinding Region)			
Tyr[1]	8.71	4.44	2.84, 2.90(CH_2) 3.42, 3.32 ($CO-CH_2-S$) 6.75, 7.03 aromatic
Apc[2]	8.59	4.38	2.80, 2.73 (CH_2) 3.03 (CH_2-NH_2) 2.44 ($-S-CH_2$) 1.88 ($CH_2-CH_2-CH_2$) 7.62 (NH_2)
Gly[3]	8.39	3.95, 4.04	
Asp[4]	8.54	4.62	2.70[a] (CH_2)
Cys[5]	8.41	4.44	3.01[b]
Gly[6]	8.56	3.98	
Gly[7]	8.36	4.01	
Cys[8]	8.43	4.62	3.00, 3.14
Gly[9]	8.62	4.01	
Cys[10]	8.13	5.99	2.88, 3.17
Acm[8]	8.69	4.31	2.02 or 1.94, CH_3
Acm[10]	8.18	4.24	2.02 or 1.94, CH_3
Binding Region			
Gly[11 or 12 c]		4.18, 4.47	
Gly[11 or 12 c]		4.41, 4.52	
Cys[13c]		5.56	3.81, 3.57
C-terminal amide	7.57, 7.24		

[a] NMR spectral data measured in 95%/5% H_2O/D_2O, pH = 4.0, T = 7 °C. [b] Methylene protons within 0.05 ppm are give the same chemical shift value. [c] For the amino acids in the binding region, no NH−α-CH coupling is observed, consistent with the loss of these protons upon binding.

of 1.2 s were employed. A mixing time of 500 ms was utilized in the NOESY and ROESY experiments, and a spin lock time of 65 ms was employed for TOCSY. For all 2D spectra, 32−64 transients with 2K points were accumulated. For the COSY spectra, 256 t_1 values were acquired. For NOESY, ROESY, and TOCSY spectra, 64 or 96 t_1 values were acquired. Data processing was carried out on a Sun LX workstation using standard Varian VNMR software. Linear prediction to either 256 or 384 points was used for data processing; the data were subsequently zero filled to 1K. The final data matrix size for all spectra was 2K × 1K. Sine bell processing was used for COSY spectra, and shifted sine bell together with baseline correction was used with TOCSY, NOESY, and ROESY.

For NMR measurements, apcitide, 1.8 mg, was dissolved in 700 μL of 95/5 H_2O/D_2O. The solution was sonicated for 20 min to effect complete dissolution of the peptide. The resulting solution was pH 4.01. For NMR of [^{99}TcO] apcitide, 2.4 mg of [^{99}TcO] apcitide was dissolved in 700 μL of 95/5 H_2O/D_2O. This solution was pH 4.0. Chemical shifts are given in Tables 1 and 2 for apcitide and [^{99}TcO] apcitide, respectively.

Preparation of [^{99}TcO] Apcitide. Apcitide (21.7 mg of the trifluoroacetate salt, 0.01247 mmol) was added to 1.5 mL of DMF with stirring at room temperature. The cloudy solution was sonicated for 30 min to dissolve all solid to form a clear solution. To the stirring, clear, colorless solution of apcitide at room temperature was added 170 μL of a stock [^{99}TcOCl$_4$] N(C_4H_9)$_4$ (7.96×10^{-2} M, 0.014 mmol) solution. Immediately, the resulting solution turned orange. The solution was stirred at room temperature. Additional DMF and [^{99}TcOCl$_4$]$^-$ and water were added over a period of a few hours. The total amounts of components added were 260 μL of [^{99}TcOCl$_4$]$^-$ (0.021 mmol), 450 μL of water, and 2.24 mL of DMF for a total of 2.95 mL of reaction solution. The reaction solution was stirred at room temperature for a period of 15 h. The reaction solution was clear and orange throughout the reaction period. The analytical HPLC, monitored during the reaction, showed that the [^{99}TcO] apcitide species coeluted with the [^{99m}TcO] apcitide tracer radiopharmaceutical.

The solution was divided into five 500 μL portions; each one was loaded onto a prep HPLC column (Delta Pak, C18) and eluted with a gradient of 14−22% B (mobile phase A = 0.1% TFA in water; B = 50/50 CH_3CN/H_2O containing 0.1% TFA) over 1 h. The peak corresponding to [^{99}TcO] apcitide was collected, and the five fractions were combined and lyophilized. Yield: 8.0 mg, 5 μmol, 42% based on peptide. This sample was characterized by NMR, mass spectrometry, and FT-IR.

Preparation of [^{99}TcO] Apcitide from Bibapcitide. The conditions of this preparation mimic the radiopharmaceutical kit conditions. To a solution of glucoheptonate (4.2 mg) and $SnCl_2 \cdot 2H_2O$ (1.5 mg), dissolved in 200 μL of sodium carbonate buffer (1 M, pH = 7), was added a solution of $NH_4{}^{99}TcO_4$ (56 μL, 0.117 M, 0.006 mmol) to form a dark pink solution. In another vial, 10 mg of bibapcitide, 0.003 mmol, was suspended in 300 μL of buffer. The ^{99}Tc glucoheptonate solution was added to the bibapcitide suspension and heated at 80 °C for 10 min to give a clear pink solution. After cooling to room temperature, the analytical HPLC showed a peak for the [^{99}TcO] apcitide that coeluted with the [^{99m}TcO] apcitide radiopharmaceutical. The reaction solution was loaded onto a prep HPLC column, and the fraction corresponding to the peak was collected, as described above, and lyophilized. Mass spectrometry data confirmed that the peak was the [^{99}TcO] apcitide species. Observed $(M-H)^-$, average m/z 1502.6 (average calcd m/z 1502.5); monoisotopic m/z 1501.6 (monoisotopic m/z 1501.4).

Results

General. The reaction proceeded rapidly at room temperature. Although we added an excess of [^{99}Tc(V)═O]$^{3+}$ reagent and stirred for 15 h for the preparative reaction, very small-scale reactions, monitored by HPLC, indicated that stoichiometric quantities and shorter reaction times would be suitable. The product was purified and isolated using preparative reversed-phase HPLC. The appropriate fraction was collected and lyophilized to give a red-coral powder. One species was observed according to HPLC and NMR measurements. [^{99}TcO] apcitide coeluted with the tracer, [^{99m}TcO] apcitide, verifying that the macroscopic [^{99}TcO]

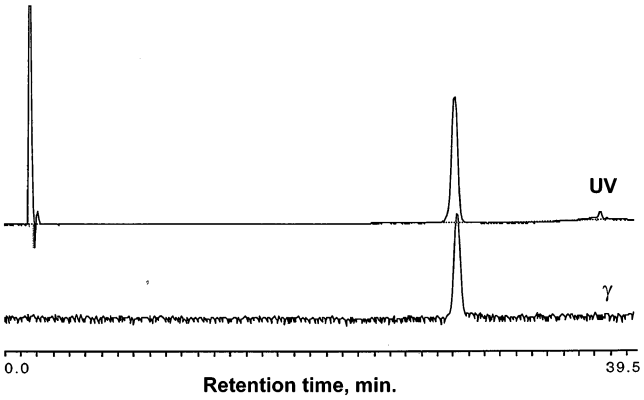

Figure 2. HPLC concordance of [99mTcO] apcitide and [99TcO] apcitide. Top trace: UV detection to monitor [99TcO] apcitide. Bottom trace: γ detection to monitor [99mTcO] apcitide. See text for HPLC conditions.

apcitide had the same chemical form as the tracer ^{99m}Tc radiopharmaceutical. The HPLC concordance is shown in Figure 2.

Under the acidic conditions used for the HPLC purification and isolation, the apc group is protonated, the aspartic acid residue is neutral, and the $[Tc^{V}O]^{3+}$ binding region bears a -1 charge (see below); therefore, the resulting species would be expected to be neutral. The NMR spectral data for both apcitide and the [99TcO] apcitide complex were collected in water at pH = 4. Under those conditions, apcitide is likely to be zwitterionic (the apc group will be protonated and the aspartate is likely to be anionic) while [99TcO] apcitide will have an overall negative charge due to the negative charge of the $[Tc^{V}O]^{3+}$ binding region.

Spectroscopy. The infrared spectra of apcitide and [99TcO] apcitide, taken as KBr pellets, are shown in Figure S1 (Supporting Information). Comparison of the apcitide and [99TcO] apcitide spectra clearly show that the Tc=O stretching frequency occurs at 961.2 cm^{-1}. This frequency is consistent with Tc=O stretches observed for anionic $[Tc^{V}O]^{3+}$ amide thiolate complexes.[15,23,24]

Mass spectral data for apcitide in the positive-ion mode show the apcitide $(M + H)^+$ monoisotopic m/z = 1392.8 ($C_{51}H_{78}O_{19}N_{17}S_5$, calcd m/z = 1392.4). An LSIMS spectrum showed $(M + H)^+$ m/z =1392.5. Fragments that correspond to loss of (acm-H) group(s) appear at m/z = 1319.8 and 1250.9 (broad, metastable) in the MALDI TOF mass spectrum and m/z = 1321.9 in the LSIMS spectrum. In the LSIMS spectrum, a peak that corresponds to loss of (acm + H) is also observed at m/z = 1319.5.

The mass spectral data, recorded in the negative ion mode, for [99TcO] apcitide show average m/z = 1502.6. [99TcO] apcitide, shown in Figure 1c, with all amino acid residues in their neutral form and a mononegative charge associated with the TcO chelate, would have the molecular formula $[C_{51}H_{73}O_{20}N_{17}S_5Tc]^-$, calcd m/z = 1503.5. The observed M^- is within the error expected with external calibration in this mass range. Thus, the mass spectral data is consistent with

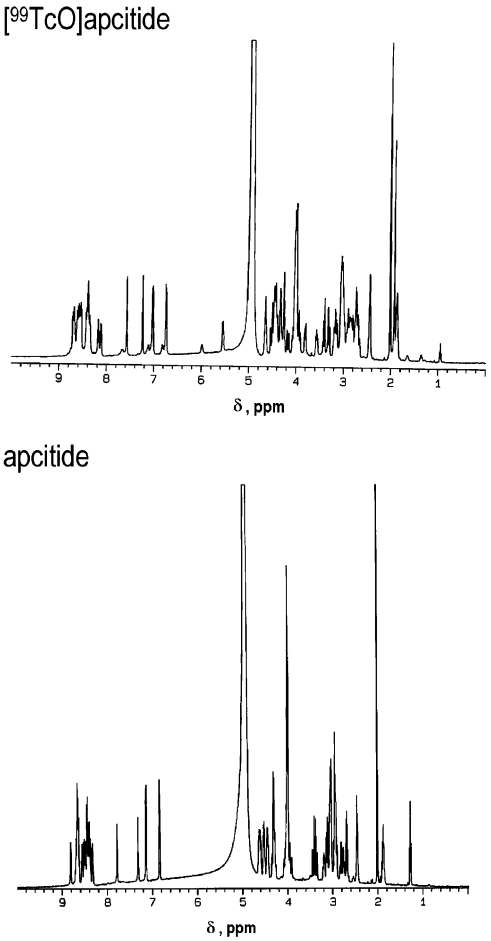

Figure 3. One-dimensional ^{1}H NMR spectroscopic data taken in 95/5 H_2O/D_2O, pH = 4, T = 7 °C. Top: [99TcO] apcitide. Bottom: apcitide.

the retention of the acm groups and the Tc binding in the Gly^{11}-Gly^{12}-Cys^{13} region of the peptide. Additional peaks corresponding to the peptide $(M - 2H + Na)^-$ ($C_{51}H_{75}O_{19}N_{17}S_5Na$) are seen at average m/z = 1414.2 (calc m/z = 1414.4), and the loss of acm-H from this species is observed at average m/z = 1343.7 (calcd average m/z = 1343.3). These additional peaks may result from fragmentation or from free apcitide present as an impurity.

NMR Spectroscopy. The strategies for assigning the resonances of apcitide and [99TcO] apcitide were similar. The spectra were recorded in 95/5 H_2O/D_2O at 7 °C. In this solvent system, the amide NH resonances can be observed. At this temperature, the H_2O signal was located at 5.00 ppm, which allows observation of all of the resonances. All spectra were referenced to the H_2O signal.

Apcitide. The one-dimensional NMR spectrum of apcitide peptide is shown in Figure 3. The chemical shift assignments, determined from the 2D experiments discussed below, are given in Table 1. Expansions of selected regions of the one-dimensional spectrum are given in Figure S2 (Supporting Information). Figure S2a gives the expansion of the α-H region. The chemical shifts for the α-hydrogen atoms for glycine residues fall in the range 3.9 to 4.1 ppm. The chemical shifts for the other α-H protons and for the acm groups fall in the 4.26 to 4.7 ppm range. The integration is appropriate for 12 α-H of the six glycines (3.90−4.15

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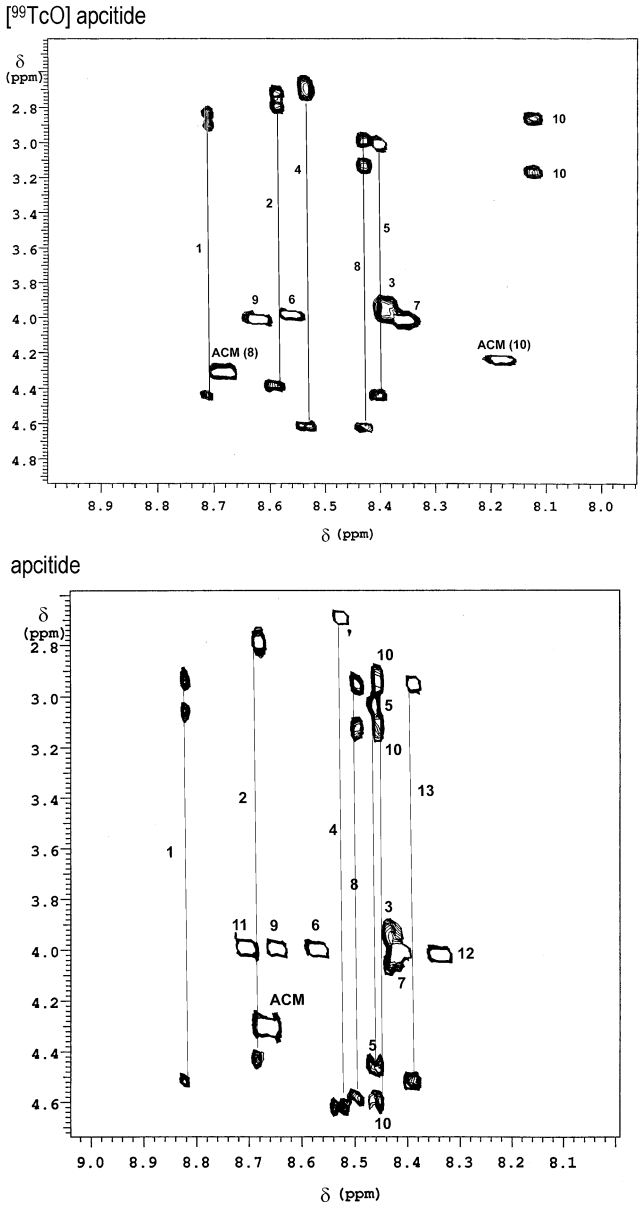

Figure 4. ^{1}H TOCSY data for peptides. See text for details. Top: [^{99}TcO] apcitide; note the NH,α-H cross-peak of Cys^{10} occurs at 5.99 ppm, 8.13 ppm. Bottom: apcitide.

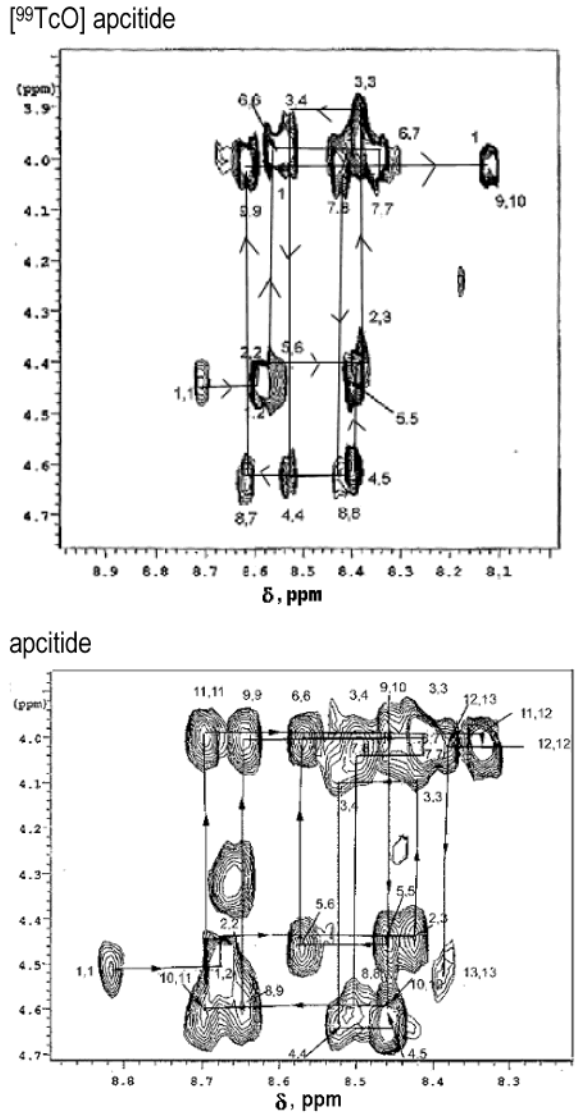

Figure 5. NOESY and ROESY spectra for peptides. See text for details. Top: NOESY data for [^{99}TcO] apcitide. Bottom: ROESY data for apcitide.

ppm): 7 α-H of other amino acids and 4 protons of the CH_2 moiety of the acm protecting group (4.26−4.70 ppm). Figure S2b shows the amide H region. Integration is appropriate for 15 NH groups (13 amino acids of the peptide plus 2 amide groups of the acm groups).

Figure 4 shows a portion of the ^{1}H TOCSY spectrum of apcitide. TOCSY provides information on the NH−α-CH interactions and the NH−X-CH_2 interactions within the same amino acid. The cross-peaks are numbered as follows: in the case where there is one cross-peak, as in the α-CH_2 of glycine amino acids, the cross-peak is numbered; in the case of the other amino acids, where there are NH−α-CH as well as NH−X-CH_2 cross-peaks, a line connects the cross-peaks and the line is numbered. The top portion of the TOCSY spectrum (2.69 ppm to 3.13 ppm) shows the NH−X-CH_2 region (within the same amino acid) for the non-glycine amino acids. As expected 7 cross-peaks are observed**.** The lower portion (3.90 ppm to 4.62 ppm) of this spectrum shows the NH−α-CH region; 6 cross-peaks are observed for the glycine amino acids, and 7 cross-peaks are observed for the non-glycine amino acids. Thus, all of the 13 amino acids, 6 glycines and 7 others, are observed in the TOCSY spectrum. The NH−CH_2 cross-peak for the acm protecting groups are also found in this region. The COSY experiment, shown in Figure S3a (Supporting Information), provides one bond connectivities, and a total count of the NH groups in the molecule can be inferred. This is important in comparing the free apcitide (13 NH groups due to amino acids and 2 NH groups of the acm groups) and [^{99}TcO] apcitide (10 NH groups due to amino acids and 2 NH groups of the acm) (vide infra). Figure S3b shows the COSY of the region from 3.2 to 4.7 ppm. The two protons of the cyclic O═C−CH_2−S−, identified by an NOE with the β-CH_2 of tyrosine, can be seen at 3.44 and 3.37 ppm. The two protons of Gly^3 are observed at 3.94 and 4.08 ppm.

Figure 5 shows the ROESY spectrum of apcitide. The ROESY and NOESY techniques are based on dipolar

interaction and show the NH−CH correlations for spatially close protons within the same amino acid and in sequential amino acids. Long-range interactions can be inferred from the NOESY/ROESY, but with the present peptides no long range NOEs were observed implying extended conformation. The starting point for the ROESY or NOESY is any amino acid that can be uniquely identified. The tyrosine residue shows a ROESY cross-peak between the β-CH_2 and the aromatic protons. From this cross-peak, the α-proton and the NH proton of the tyrosine could be identified, via the TOCSY experiment, and this served as an unambiguous starting point for sequential assignments. Sometimes due to particular motion of the peptide, certain NOESY peaks are not observed. In this case, the spatial interactions can generally be detected by the related ROESY technique.

The ROESY spectrum shows considerable overlap of signals because most of the residues of the peptide are glycines and cysteines (or derivatives). However, there is enough chemical shift dispersion to make assignments. On this spectrum, similar to a NOESY spectrum, one can "walk" through the sequence from amino acids 1 to 13 following the NH−CH NOEs within the same amino acid and between sequential amino acids. The acm NH−CH NOEs are seen in the ROESY spectrum. These mobile side chains were not detected using the NOESY technique, but in all other aspects the NOESY spectrum was very similar to the ROESY spectrum.

[^{99}TcO] Apcitide. From the one-dimensional NMR, shown in Figure 3, it is evident from the tyrosine resonances at 6.8 and 7.2 ppm that there is a small amount of an impurity in the sample. Based on chemical shifts, this may be the free peptide or a related species. The impurity did not affect the assignment of the major species by the combination of NMR techniques used. It may, however, have a minimal effect on the integration of the one-dimensional spectrum. The one-dimensional NMR spectrum also shows resonances at 0.9 ppm, 1.4 ppm, 1.7 ppm, and 3.2 ppm, all connected as seen in the COSY spectrum. These are very likely due to a small amount of tetrabutylammonium ion impurity from the ^{99}Tc starting material that may have carried through the preparation.

Also, from the one-dimensional NMR, the methyl groups of the acm protecting groups were clearly present. Two resonances are seen at 1.94 ppm and 2.02 ppm for the complex consistent with the [Tc^{V}O] binding in the adjacent Gly^{11}-Gly^{12}-Cys^{13} region. Also, new resonances at 5.56 and 5.99 ppm (broad) are present in the one-dimensional spectrum of [^{99}TcO] apcitide. This is consistent with the α-H's of some of the amino acids in the metal binding site or close to the binding site shifting downfield upon complexation of the metal. This finding was confirmed in the subsequent studies. Expansions of the one-dimensional NMR are given in Figure S4a (α-CH and acm region) and in Figure S4b (downfield region) (Supporting Information). The integration is consistent with 12 NH protons (10 from the amino acids and 2 from the acm protecting groups in the range of δ 8.0 to 8.8 ppm) compared to 15 NH protons, seen in this region for the peptide without technetium. However, due to uncertainty of integration, the definitive count of NH protons comes from the 2D experiments discussed below.

Figure 4 shows the region of the TOCSY spectrum displaying NH connectivities of [^{99}TcO] apcitide. The COSY spectrum for the same region is shown in Figure S5a (Supporting Information). Nine amino acids are seen in the NH−α-CH region, four glycines and five other amino acids. The αH of amino acid 10, the acm protected Cys^{10}, adjacent to the binding site, is found at 5.99 ppm, and the NH of Cys^{10} is found at 8.13 ppm, so the NH−CH cross-peak does not occur in the region of the figure. The NH−α-CH region of Cys^{10} can be seen in the COSY spectrum in Figure S5b. The NH−CH_2 cross-peaks of the two acm groups can be seen at 8.69 ppm, 4.31 ppm (Cys^{8}) and 8.18 ppm, 4.24 ppm (Cys^{10}). Thus, cross-peaks for the first 10 amino acids are observed as expected if the NH groups of three amino acids (Gly^{11}, Gly^{12}, Cys^{13}) are deprotonated upon complexation to the $[TcO]^{3+}$ group.

The α-protons from Gly^{11} and Gly^{12} are found shifted outside the "usual" glycine proton range of 3.99 to 4.01 ppm, as is often found when a metal ion binds to amide groups.[25−29] Figure S5c shows the COSY spectrum of the α-proton region of Gly^{11} and Gly^{12}. The cross-peaks observed at 4.47 ppm, 4.18 ppm and 4.41 ppm, 4.52 ppm are assigned to Gly^{11} and Gly^{12}; these cannot be distinguished. However these resonances show no cross-peaks in the NH region of the TOCSY and COSY, consistent with Gly^{11} and Gly^{12} as part of the Tc binding site. Also in Figure S5c, the protons of Cys^{13} are observed at 3.81 and 3.57 ppm. The COSY spectrum of this region of [^{99}TcO] apcitide should be contrasted to that of apcitide (Figure S3b), where downfield shifts of the Gly^{11}, Gly^{12}, and Cys^{13} are not observed.

Figure 5 shows a region of the NOESY spectrum of [^{99}TcO] apcitide. On this spectrum, one can "walk" through the sequence from amino acids 1 to 10 following the NH−CH NOEs within the same amino acid and between sequential amino acids. Amino acids at the end of the peptide, Gly^{11}, Gly^{12}, Cys^{13}, in the binding region, have no NH protons; therefore, no NOEs between NH−CH are observed after Cys^{10}. The acm NH−CH_2 cross-peaks which were not seen in the NOESY were clearly seen using the ROESY technique. Also in the ROESY spectrum, one can see cross-peaks from the CH_2 of the acm group to the CH_2 of the cysteinyl moiety that allows assignments of the methylene protons of the acm to a particular amino acid. No cross-

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peaks were seen to the methyl groups of the acm units; therefore the 2 methyl groups could not be specifically assigned.

Discussion

Despite significant spectral overlap due to numerous similar amino acids, all protons of apcitide and [^{99}TcO] apcitide were unambiguously assigned. All chemical shifts are within the range expected for the constituent amino acids in both peptides.

There are several significant differences between apcitide and [^{99}TcO] apcitide. Among these are (1) the nonequivalence in chemical shifts of the two acm groups in [^{99}TcO] apcitide; (2) the presence of only 10 NH−CH cross-peaks in both the COSY and TOCSY spectra of the Tc complex compared to 13 found in apcitide; the absence of NH−CH cross-peaks for Gly^{11}, Gly^{12}, Cys^{13} in the technetium complex; (3) the downfield shift and splitting of the α-methylene proton of the two glycine residues (Gly^{11}, Gly^{12}) of [^{99}TcO] apcitide compared to apcitide; (4) the downfield shifts of the α-methine protons in Cys^{13} and Cys^{10} in [^{99}TcO] apcitide compared to the uncomplexed peptide; (5) the downfield shift and large chemical shift nonequivalence of the β-CH$_2$ protons of Cys^{13} in the ^{99}Tc complex. The NOESY and ROESY spectra confirm the amino acid sequences in apcitide and [^{99}TcO] apcitide.

All of the above are indicative of binding of the [Tc^{V}═O]$^{3+}$ unit to the deprotonated amide nitrogen atoms of Gly^{11}, Gly^{12}, Cys^{13} and the thiolate sulfur of Cys^{13}. Deprotonation of amine and amide groups upon complexation to Tc^{V}═O and Re^{V}═O has been observed for amine oxime ligands,[30,31] amine thiol and amide thiol ligands.[24−28,32,33] In crystallographic studies, the deprotonated "amide" nitrogen atoms form short (ca. 1.90−1.96 Å) bonds to the Tc indicative of multiple bonding character. NMR studies, usually performed in an organic solvent, clearly indicate the absence of the proton. By performing our study in H_2O/D_2O, we can clearly observe the amide protons and thus accurately assess the loss of three protons from Gly^{11}, Gly^{12}, and Cys^{13} upon complexation of apcitide with Tc. NMR studies performed in H_2O/D_2O were critical to establish that one amide nitrogen and three thiolate sulfur atoms comprised the binding site in Re α-melanotropin peptide analogues.[34] NMR studies of the water soluble tetrapeptide complexes of Tc and Re, ^{99}TcO[RP290] and ReO[RP290], were performed in D_2O, where exchange with deuterium would broaden out any amine or amide protons.[16]

The FT infrared data shows a Tc═O stretch (961.2 cm^{-1}) in the range observed for anionic [Tc^{V}O]$^{3+}$ amide thiolate complexes.[15,23,24] A structure where the [Tc^{V}O]$^{3+}$ moiety is incorporated into the Gly^{11}-Gly^{12}-Cys^{13} region, as in Figure 1, would give an anionic Tc binding region. The mass spectral data also agrees with the structure shown in Figure 1. The molecular weight is appropriate for the molecule with the retention of the acm groups and the binding of the metal in the Gly^{11}-Gly^{12}-Cys^{13} region.

The macroscopic reaction mimicking radiopharmaceutical kit conditions, that is the reaction of ^{99}TcO$_4^-$ with bibapcitide, in the presence of Sn(II) and glucoheptonate, with heat, shows one species that coelutes with the [^{99m}TcO] apcitide radiopharmaceutical. Mass spectrometry confirms that the chemical formula is identical to the species formed upon reaction of the ^{99}Tc^{V}O starting material, ^{99}TcOCl$_4^-$, with apcitide. In both cases, it is clear that the acm groups are retained, the bis-succinimidomethyl ether linkage is broken in bibapcitide, and the ^{99}Tc binds in the Gly^{11}-Gly^{12}-Cys^{13} region to form [^{99}TcO] apcitide.

The thiol groups of Cys^{8} and Cys^{10} are capped by the acetamidomethyl (acm) protecting groups in the peptides, apcitide and bibapcitide. Both acm groups are retained in the Tc complexes, at the macroscopic level when either ^{99}TcOCl$_4^-$ or ^{99}TcO$_4^-$ and Sn(II) are used to form [^{99}TcO] apcitide, and at the tracer level in the formation of [^{99m}TcO] apcitide. The acm protecting group is a common method to protect thiolates,[32,35,36] as it is readily removed upon reaction with ^{99m}TcO$_4^-$ and ^{99}TcO$_4^-$ and stannous ion under neutral to basic conditions[16] and with [^{99}Tc^{V}O]$^{3+}$ reagents in organic or organic/aqueous solvents. However, in the reaction of apcitide with ^{99}TcOCl$_4^-$ at room temperature and bibapcitide with macroscopic ^{99}TcO$_4^-$ and stannous ion at 80 °C, it is clear that the acm groups remain intact and the ^{99}Tc binds in the gly gly cys region. The $(N_{amide})_3S_{thiol}$ donor atoms of Gly^{11}, Gly^{12}, Cys^{13} form a stable bonding sequence, and, given that this sequence is located in a position remote from the rigid cyclic portion of the molecule, it is not surprising that the Gly^{11}, Gly^{12}, Cys^{13} sequence is a more favorable binding region compared with the Cys^{8}, Gly^{9}, Cys^{10} sequence. Moreover, metal binding in the Cys^{8}, Gly^{9}, Cys^{10} sequence would result in one six-membered chelate ring formed by binding to the Cys^{8} and two five-membered rings, that would probably not be as stable as the three five-membered rings formed by the Gly^{11}, Gly^{12}, Cys^{13} motif.

Reverse phase HPLC is the best method to study these Tc peptides. Ion exchange chromatography did not yield any useful information that would distinguish between the cys-gly-cys binding site or the gly-gly-cys binding site. In fact, if the former were the metal binding site and the dimeric formulation were retained, one would expect the species to be highly retained on the reverse phase column and elute at a retention time close to that of the bibapcitide peptide, ca. 65 min, using the analytical system described previously. This is clearly not the case. Further confirmation that the ^{99m}TcO apcitide radiopharmaceutical has the structure of

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Figure 1 is derived from the mass spectrometry of the species isolated from the reaction that mimics the radiopharmaceutical kit conditions where bibapcitide is reacted with $^{99}TcO_4^-$, glucoheptonate, and Sn(II) at 80 °C.

One species was observed in the reaction of Tc with apcitide to form a Tc^VO complex. In most tetradentate chelating ligands with a chiral carbon or an N-alkylated moiety, diastereomers are often observed when a $[Tc^V{=}O]^{3+}$ group is bound to the donor atoms with square pyramidal coordination geometry. Pendant groups are disposed in either syn or anti positions relative to the Tc═O group.[16,25,26] In some cases, one isomer was selectively formed over another.[37] In the study of Tc and Re complexes of dimethylglycyl-L-seryl-L-cysteinylglycinamide (RP294), syn and anti isomers, considering the serine side chain, formed in close to 1:1 ratio, according to NMR studies.[16]

There have been a few preliminary studies on ^{99m}Tc complexes of tetra- and hexapeptides where one species has been observed even though the ligand is composed of chiral amino acids. In a study of N_4 tetrapeptides, direct labeling of the tetrapeptides with $^{99m}TcO_4^-$ in alkaline medium (pH ≥ 11) in the presence of stannous ions gave a high yield of one radiochemical species, if the chiral amino acid was in the second or third amino acid. Two radiochemical species, presumably syn and anti isomers, were found when the chiral amino acid was the first amino acid.[38] Chirality around the Tc═O group in [^{99}TcO] apcitide is introduced in the Cys^{13} (third) amino acid; the formation of one species is consistent with observations found for the N_4 tetrapeptides.

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Conclusion

We report the characterization of the ^{99}Tc form of the radiopharmaceutical [^{99m}TcO] apcitide (AcuTect). The ^{99}Tc complex of the 13 amino acid peptide, apcitide, has been prepared, on the macroscopic level, by reaction of $[^{99}TcOCl_4]^-$ with apcitide or by reaction of $^{99}TcO_4^-$ and Sn(II) with bibapcitide, purified by preparative HPLC and isolated as a trifluoroacetate salt. The NMR spectral data clearly show that the $[TcO]^{3+}$ unit is bound to the $Gly^{11}Gly^{12}Cys^{13}$ region at the C-terminal end of the peptide. Both acm groups are retained upon complexation. Mass spectral data and FT IR data are consistent with the NMR data.

The results of these NMR experiments demonstrate that a significant amount of structural information can be obtained from NMR studies of these types of complexes. Through NMR techniques, peptide sequence and metal chelate regions can be confirmed.

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Supporting Information Available: Figures S1 through S5. This material is available free of charge via the Internet at http://pubs.acs.org.

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