Tuftsin Receptor-Binding Peptide Labeled with Technetium: Chemistry and Preliminary in Vitro Receptor-Binding Study

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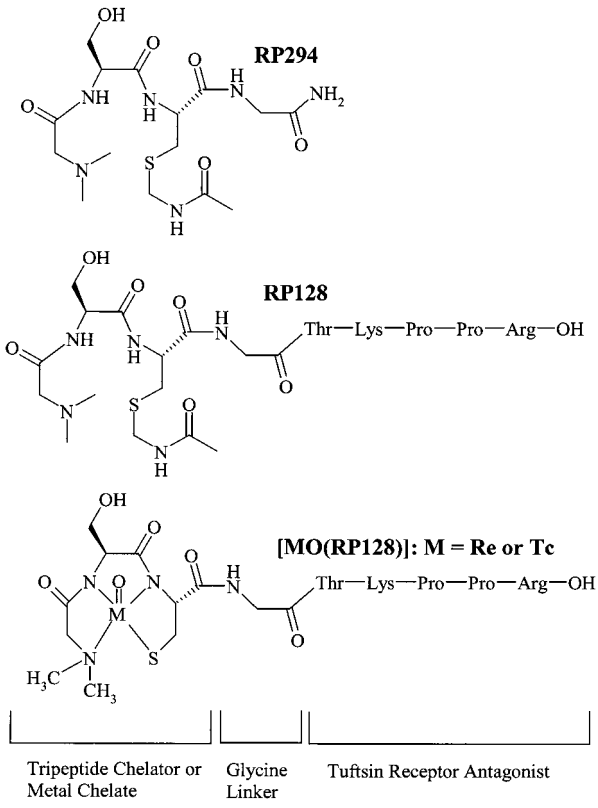
Introduction

Technetium-99m (^{99m}Tc) is the radionuclide of choice in diagnostic nuclear medicine. The advantages of using ^{99m}Tc in nuclear medicine are well-known, and a number of review papers describing the development of technetium-based imaging agents have been published in recent years.[1−12] A particular area of interest has been the design of target specific radiopharmaceuticals using the bifunctional approach in which a ^{99m}Tc chelate is connected via an appropriate linker to a known targeting molecule.

The technetium complex of dimethylglycyl-L-seryl-L-cysteinyl(acm)-glycyl-L-threonyl-L-lysyl-L-prolyl-L-prolyl-L-arginine (RP128) (Chart 1) was designed via the bifunctional approach as an inflammation imaging agent targeting the tuftsin receptor.[13−16] Tuftsin, threonyl-lysyl-prolyl-arginine (TKPR), is a natural immunostimulant derived from IgG through proteolytic cleavage in the spleen and by a neutrophile-derived enzyme (leukokininase). It stimulates a number of functional responses by phagocytes including phagocytosis, respiratory burst, and antigen presentation.[17−21] The targeting component of RP128 is the tuftsin receptor antagonist threonyl-L-lysyl-L-prolyl-L-prolyl-L-arginine (TKPPR),[22−25] which has a 4-fold greater receptor affinity than tuftsin.[19,25]

The results from a phase I clinical study evaluating ^{99m}Tc RP128 in both healthy volunteers and rheumatoid arthritis patients were reported recently.[16] Though it is suspected that RP128 would be coordinated to a Tc oxo moiety via the deprotected dimethylglycyl-L-seryl-L-cysteine component of the molecule, no chemical characterization had been reported to date. It was our goal to synthesize and characterize ^{99}Tc and Re complexes of RP128 in order to confirm the site of metal coordination. It was also our desire to determine whether the attachment of the technetium chelate-linker would affect the conformation or binding affinity of the TKPPR targeting moiety.

Chart 1



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(1) Liu, S.; Edwards, D. S. *Chem. Rev.* **1999**, *99*, 2235.
(2) Jurisson, S. S.; Lydon, J. D. *Chem. Rev.* **1999**, *99*, 2205.
(3) Seregni, E.; Chiti, A.; Bombardieri, E. *Eur. J. Nucl. Med.* **1998**, *25*, 639.
(4) Schmidt, M.; Scheidhauer, K.; Luyken, C.; Voth, E.; Hildebrandt, G.; Klug, N.; Schicha, H. *Eur. J. Nucl. Med.* **1998**, *25*, 675.
(5) Pauwels, E. K.; McCready, V. R.; Stoot, J. H. M. B.; van Deurzen, D. F. P. *Eur. J. Nucl. Med.* **1998**, *25*, 277.
(6) Liu, S.; Edwards, D. S.; Barrett, J. A. *Bioconjugate Chem.* **1997**, *8*, 621.
(7) Lister-James, J.; Moyer, B. R.; Dean, R. T. Q. *J. Nucl. Med.* **1997**, *41*, 111.
(8) Hom, R. K.; Katzenellenbogen, J. A. *Nucl. Med. Biol.* **1997**, *24*, 485.
(9) Eckelman, W. C.; Steigman, J.; Paik, C. In *Diagnostic Nuclear Medicine*, 3rd ed.; Williams & Wilkins: Baltimore, 1996; pp 199−216.
(10) Archer, C. M.; Edwards, B.; Powell, N. A. In *Current Directions in Radiopharmaceutical Research and Development*; Mather, S. J., Ed.; Kluwer Academic Publishers: The Netherlands, 1996; pp 81−88.
(11) Lister-James, J.; Moyer, B. R.; Dean, R. T. Q. *J. Nucl. Med.* **1996**, *40*, 221.
(12) Thakur, M. L. *Nucl. Med. Commun.* **1995**, *16*, 724.
(13) Cavelier, V.; Goodbody, A. E.; Tran, L. L.; Bossuyt, A.; Thornback, J. *Eur. J. Nucl. Med.* **1996**, *23*, 1131 (abstract Omo398).
(14) Peers, S. H.; Tran, L. L.; Eriksson, S. J.; Ballinger, J.; Goodbody, A. E. *J. Nucl. Med.* **1995**, *36*, 114P (abstract 465).
(15) Paul, C.; Peers, S. H.; Woodhouse, L. E.; Thornback, J. R.; Goodbody, A. E.; Bolton, C. *J. Neurosci Methods*, **2000**, *98*, 83.
(16) Caveliers, V.; Goodbody, A. E.; Tran, L. L.; Peers, S. H.; Thornback, J. R.; Bossuyt, A. *J. Nucl. Med.* **2001**, *42*, 154.
(17) Klegeris, A.; McGeer, P. L. *J. Neuroimmunol.* **1994**, *53*, 83−90.
(18) Kraus-Berthier, L.; Ferry, G.; Combe-Perez, V.; Visalli, M.; Remond, G.; Vincent, M.; Boutin, J. A. *Biochem. Pharmacol.* **1991**, *41*, 1411.
(19) Bump, N. J.; Lee, J.; Wleklik, M.; Reichler, J.; Najjar, V. A. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 7187.
(20) Dagan, S.; Gottlieb, P.; Fridkin, M.; Spirer, Z.; Tzehoval, E.; Feldman, M. *Receptors* **1986**, *3*, 243.
(21) Chaudhuri, M. K.; Konopinska, D.; Bump, N. J.; Najjar, V. A. *Ann. N. Y. Acad. Sci.* **1983**, *419*, 135.
(22) Gershonov, E.; Granoth, R.; Tzehoval, E.; Gaoni, Y.; Fridkin, M. *J. Med. Chem.* **1996**, *39*, 4833.
(23) Nishioka, K.; Satoh, P. S.; Constantopoulos, A.; Najjar, V. A. *Biochim. Biophys. Acta* **1973**, *310*, 230.
(24) Fridkin, M.; Gottlieb, P. *Mol. Cell. Biochem.* **1981**, *41*, 73.
(25) Bump, N. J.; Najjar, V. A.; Reichler, J. *Mol. Cell. Biochem.* **1990**, *29*, 77.

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In this paper, we report the synthesis and characterization of RP128 and its technetium and rhenium analogues. The binding affinity of TKPPR and the rhenium complex of RP128 to the tuftsin receptor were also compared.

Experimental Section

Materials. $[ReO_2(en)_2]Cl$, where en is ethylenediamine, was prepared according to literature methods.[26,27] Mercury(II) acetate, *tert*-butyl methyl ether, and trifluoroacetic acid were purchased from Aldrich Chemicals, Inc. Sephadex G10 resin, tin(II) chloride, and sodium gluconate were purchased from Sigma Chemical Co. The peptide threonyl-L-lysyl-L-prolyl-L-prolyl-L-arginine (TKPPR), sasrin resin (2-methoxy-4-alkoxybenzyl alcohol resin), and 9-fluorenylmethyloxycarbonyl (FMOC)-protected amino acids were purchased from Bachem Bioscience, Inc. 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBT), piperidine, *N*,*N*-dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), and diisopropylethylamine (DIEA) were purchased from Applied Biosystems, Inc. Tritated threonyl-L-lysyl-L-prolyl-L-arginine was prepared by NEN Life Science by catalytically reducing threonyl-L-lysyl-L-dehydroproline-L-arginine with tritium. The tritated threonyl-L-lysyl-L-prolyl-L-arginine was characterized by HPLC and mass spectrometry. The HPLC retention times of the tritated threonyl-L-lysyl-L-prolyl-L-arginine were consistent with the threonyl-L-lysyl-L-prolyl-L-arginine standard (t_R = 5.2 min using method C), and the mass spectral data were consistent with a tritated threonyl-L-lysyl-L-prolyl-L-arginine. $Na[^{99m}TcO_4]$ was purchased from Mallinckrodt, Inc., and was eluted from a $^{99}Mo/^{99m}Tc$ generator and delivered on the day of the experiment. $NH_4[^{99m}TcO_4]$ was obtained from the research laboratory of the late Professor Colin Lock of McMaster University and was obtained originally from AECL, Inc. All chemicals were used as received.

Caution! ^{99}Tc is a low-energy (0.292 MeV) β^- emitter with a half-life of 2.12×10^5 years. ^{99m}Tc is a γ emitter (140 keV) with a half-life of 6.01 h. All manipulations of solutions and solids were performed in a laboratory approved for the handling of radioisotopes, and normal safety procedures were followed at all times to prevent contamination.

Instrumentation. NMR spectra were recorded on a Bruker Advance DRX-500 spectrometer. ^1H NMR spectra were acquired at 500.130 MHz with a 5 mm broad band inverse probe with triple axis gradient capability. Spectra were obtained in eight scans in 32 K data points over a 4.006 kHz spectral width (4.096 s acquisition time). Sample temperature was maintained at 30 °C by a Bruker Eurotherm variable temperature unit. Gaussian multiplication (line broadening: −1.5 Hz, Gaussian broadening: 0.2) was used to process the free induction decay (FID), which was zero-filled to 64 K before Fourier transformation. Coupling constants (*J*) were reported in Hz.

^{13}C NMR spectra were recorded at 125.758 MHz with a 5 mm broad band inverse probe with triple axis gradient capability. The spectra were acquired over a 28.986 kHz spectral width in 32K data points (0.557 s acquisition time). The ^{13}C pulse width was 4.0 μs (30° flip angle). A relaxation delay of 0.5 s was used. Exponential multiplication (line broadening: 4.0 Hz) was used to process the FID, which was zero-filled to 64K before Fourier transformation.

Inverse detected ^1H-^{-3}C two-dimensional chemical shift correlation spectra were acquired in the phase-sensitive mode and used the pulsed field gradient version of the HSQC pulse sequence. The FIDs in the F2 (^1H) dimension were recorded over a 3.655 kHz spectral width in 1 K data points. The 128 FIDs in the F1 (^{13}C) dimension were obtained over a 21.368 kHz spectral width. Each FID was acquired in the two scans. The fixed delays during the pulse sequence were 1.0 s relaxation delay and a polarization transfer delay of 1.786 μs. The 90° ^1H pulse was 6.6 μs while the ^{13}C 90° pulse was 11.6 μs. The data were processed with a sine-bell squared window function shifted by $\pi/2$ in both dimensions and linear prediction to 256 data points in F1 followed by zero-filling to 1 K.

The pulsed field gradient version of the HMBC pulse sequence was used to acquire the inverse detected $^1H-^{13}C$ two-dimensional chemical shift correlation spectra through two and three-bond coupling interactions in the absolute value mode. The FIDs in the F2 (^1H) dimension were recorded over a 3.655 kHz spectral width in 1K data points. The 128 FIDs in the F1 (^{13}C) dimension were obtained over a 21.368 kHz spectral width. Each FID was acquired in two scans. The fixed delays during the pulse sequence were a 1.0 s relaxation delay, a 3.3 ms delay for the low pass *J* -filter, and 0.08 s delay to allow evolution of the long-range coupling. The 90° ^1H pulse was 6.6 μs, while the ^{13}C 90° pulse was 11.6 μs. The data were processed with a sine-bell window function in both dimensions and linear prediction to 256 data points in F1 followed by zero-filling to 1K.

Compounds studied by NMR were dissolved in the appropriate deuterated solvents to a concentration of approximately 15.0 mg/mL whenever possible. Chemical shifts are reported in ppm relative to TMS. The residual solvent signals were used as internal references for the ^1H and ^{13}C NMR spectra, respectively.

Electrospray ionization mass spectrometry was performed with 50/50 CH_3CN/H_2O as the mobile phase at a flow rate of 15 mL per minute, with the use of a Brownlee Microgradient syringe pump. Samples were dissolved in 50/50 CH_3CN/H_2O with the addition of 1 drop of 0.1% TFA for samples that were to be analyzed in the positive mode. Full scan ESMS experiments were performed with a Fisons Platform quadrupole instrument.

The HPLC analyses and purification of all Re complexes were performed using a Beckman System Gold Nouveau chromatographic system with a Waters 4 mm × 250 mm radial pak C-18 column. During analytical HPLC analysis (HPLC method A), the mobile phase was changed from 100% water/0% acetonitrile to 0% water/100% acetonitrile over 20 min at a flow rate of 2 mL/min. Both the water and acetonitrile solutions contained 0.1% trifluoroacetic acid. The HPLC analyses were monitored with a UV detector (Beckman system model 168 detector) set at 215 nm. Purification of the Re complex of RP128 was performed using a step isocratic method (method B) with the mobile phase changing from 100% water/0% acetonitrile to 91% water/9% acetonitrile over 5 min, followed by a change in the mobile phase from 91% water/9% acetonitrile to 88% water/12% acetonitrile over 18 min at 3 mL/min.

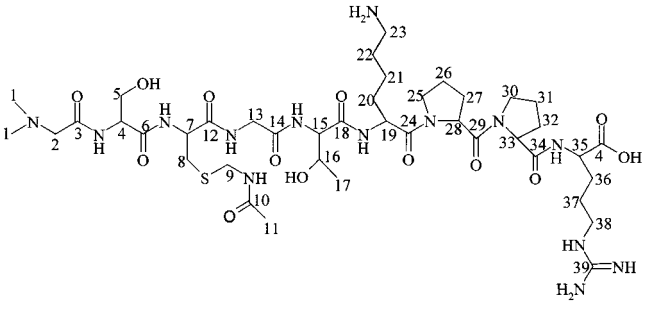
HPLC analyses of ^{99m}Tc samples were made on a Beckman System Gold chromatographic system with a Vydac 4.6 mm × 250 mm radial pak C-18 column. Two methods were used to analyze the ^{99m}Tc samples (method C and D). In method C, the mobile phase was changed from 100% water/0% acetonitrile to 30% water/70% acetonitrile over 25 min at a flow rate of 1 mL/min. In method D, the mobile phase changed from 100% water/0% acetonitrile to 70% water/30% acetonitrile over 45 min at a flow rate of 1 mL/min. The HPLC analyses of the ^{99m}Tc samples were monitored with a UV detector (Beckman system model 168 detector) set at 215 nm and a radiometric γ detector (Beckman system model 171 radioisotope detector).

RP128 was analyzed and purified on a Waters 625 Liquid Chromatograph System equipped with a Waters 717 autosampler, a Waters 996 photodiode array detector, and a Vydac 4.6 mm × 250 mm radial pak C-18 column. The method (method E) used to purify RP128 was an isocratic method set at 90% water/10% acetonitrile over 25 min. In the analytical analysis of RP128 (method F), the mobile phase changed from 100% water/0% acetonitrile to 30% water/70% acetonitrile over 20 min at a flow rate of 1 mL/min.

Synthesis of Dimethylglycyl-L-seryl-L-cysteinyl(acm)-glycyl-L-threonyl-L-lysyl-L-prolyl-L-prolyl-L-arginine (RP128). RP128 was prepared via a solid-phase peptide synthetic method[28a] on an Applied Biosystems, Inc., model 433A peptide synthesizer using FMoc 1.0 mmol chemistry.[28b] Preloaded FMOC arginine sasrin resin and FMOC amino acid derivatives were used. Prior to the addition of each amino acid residue to the N-terminus of the peptide chain, the FMOC group was removed with 20% piperidine in NMP. Each FMOC amino acid residue was activated with 0.50 M HBTU and HOBT in DMF, in the presence

(26) Rouschias, G. *Chem. Rev.* **1974**, *74*, 531.
(27) Fergusson, J. E. *Coord. Chem. Rev.* **1966**, *1*, 459.
(28) (a) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149. (b) *User's Manual*; SynthAssist 2.0, Version 2.0 Applied Bioscience Inc., Philadelphia, 1990.

Table 1. ^{1}H and ^{13}C NMR Spectral Data for RP128



^{1}H assignments	δ (ppm)	^{13}C assignments	δ (ppm)
H-7, H-28 or H-33	4.600	C-18, C-6, C-14, C-40, or C-12	175.23
H-19	4.517	C-10	174.32
H-4	4.448	C-34 or C-29	174.18
H-28 or H-33	4.327	C-12	172.46
H-9, H-35, H-15	4.235−4.206	C-34 or C-29	172.19
H-16	4.055	C-18, C-6, C-14, C-40, or C-12	171.58
H-2	3.972	C-18, C-6, C-14, C-40, or C-12	171.51
H-13a	3.922	C-18, C-6, C-14, C-40, or C-12	171.36
H-13b	3.867	C-18, C-6, C-14, C-40, or C-12	171.34
H-5	3.760	C-3	165.61
H-25 or H-30	3.732	C-39	156.83
H-25 or H-30	3.524	C-16	67.23
H-38	3.097	C-5	61.10
H-8a	2.973	C-28 orC-33	60.28
H-23	2.859	C-15	58.99
H-8b	2.835	C-28 orC-33	58.85
H-1	2.834	C-2	58.14
H-32 or H-27	2.228	C-4	55.50
H-32 or H-27	2.179	C-7	53.49
H-31, H-26	1.907	C-35	52.39
H-11	1.889	C-19	51.41
H-36, H-20	1.818	C-25 or C-30	47.99
H-36	1.678	C-25 or C-30	47.81
H-37, H-22	1.557	C-1	43.86
H-21	1.348	C-13	42.60
H-17	1.060	C-9	40.90
		C-38	40.51
		C-23	39.28
		C-8	31.59
		C-20	29.89
		C-27 or C-32	29.28
		C-27 or C-32	28.24
		C-36	27.80
		C-22	26.34
		C-26 or C-31	24.67
		C-26 or C-31	24.58
		C-37	24.37
		C-11	22.08
		C-21	21.89
		C-17	18.92

of 2.0 M DIEA in NMP. The peptide was cleaved off the sasrin resin using 95% trifluoroacetic acid. The sasrin resin was removed by filtration. The addition of the filtrate to *tert*-butyl methyl ether at 0 °C caused the precipitation of the crude product. The crude RP128 product was purified by HPLC using method E. Anal. Calcd (found) for $C_{41}H_{73}N_{14}O_{13}S_1$: C, 49.14 (49.32); H, 7.34 (7.19); N, 19.57 (19.49). Mass spectrum (electrospray): $m/z = 1001$ ($[MH^+, C_{41}H_{73}N_{14}O_{13}S_1]^+$). HPLC retention time: $t_R = 5.8$ min using method F. ^{1}H and ^{13}C NMR spectral data for RP128 are listed in Table 1.

Synthesis of the Rhenium Oxo Complex of RP128 ([ReO-(RP128)]). RP128 (204 mg, 0.204 mmol) was dissolved in 2 mL of 30% acetic acid. Mercury(II) acetate (133 mg, 0.417 mmol) was added to the solution, and the solution was stirred under Ar at room temperature for 6 h. H_2S gas was then bubbled through the solution for 5 min, causing black HgS to precipitate. The precipitate was removed by vacuum filtration, and the filtrate was frozen and lyophilized overnight. The resulting deprotected RP128 was used immediately in the subsequent metal complexation reactions.

$[ReO_2(en)_2]Cl$ (50.5 mg, 0.135 mmol) was dissolved in 1.5 mL of distilled water. Deprotected RP128 (100 mg, 0.107 mmol) was dissolved in 2 mL of distilled water. The solutions were combined to give a light green solution. The pH of the solution was adjusted to 6 using 1 M NaOH. The solution was refluxed under Ar for 1 h, during which time the solution changed from green to red. The solution was frozen and lyophilized, yielding a red solid. Purification of the product was done by HPLC using method B. Yield: 79 mg (52%). Anal. Calcd (found) for $C_{39}H_{65}N_{13}O_{13}Re_1S_1$: C, 41.01 (40.97); H, 5.74 (5.68); N, 15.94 (15.88). Mass spectrum (electrospray): $m/z = 1130$ (MH^+, $[C_{38}H_{66}N_{13}O_{13}Re_1S_1]$). HPLC retention time: $t_R = 6.1$ min using method A. HPLC retention time using method D: ReO(RP128) $t_R = 18.26$, 18.85 min. IR (KBr disk): 980 cm^{-1} ($\gamma_{Re=O}$).

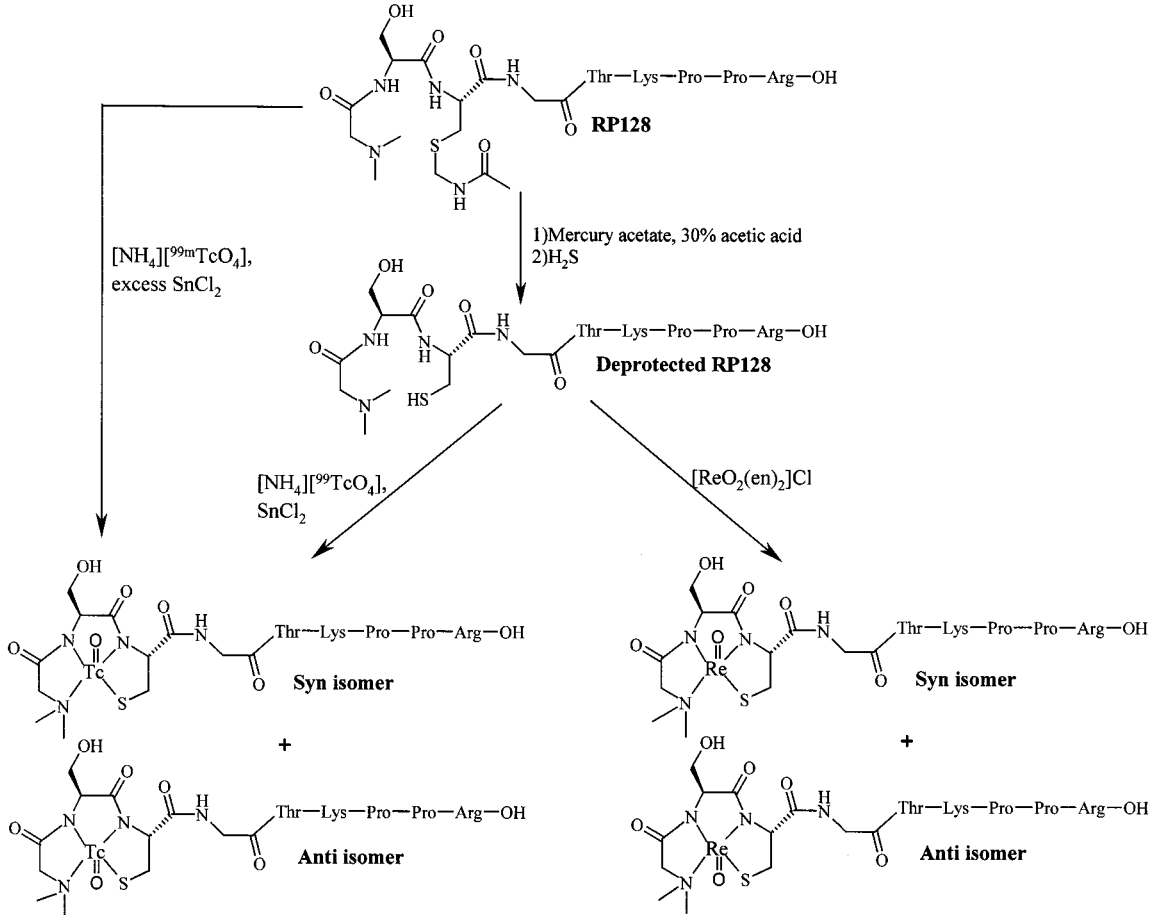
Synthesis of the ^{99}Tc Oxo Complex of RP128 ([^{99}TcO(RP128)]). The acetoamidomethyl protection group was removed in the same manner as described in the synthesis of the Re oxo RP128 complex. Deprotected RP128 (20 mg, 0.021 mmol) was dissolved in 2 mL of dry pyridine. Tin(II) chloride (4.6 mg, 0.024 mmol) and NH_4TcO_4 (3.3 mg, 0.018 mmol) in 1 mL of dry pyridine were added to the peptide solution. The solution was stirred at room temperature for 1 h. The color of the solution changed to orange-red. The solution was frozen and lyophilized overnight, yielding a red solid. The product was purified using a 1 cm × 15 cm column packed with Sephadex G10 resin and washed with 20 mL of distilled deionized water. The crude product was loaded onto column and eluted with distilled deionized water. Twenty fractions of 2 mL each were collected and the orange-pink fractions were evaporated to dryness and examined by NMR spectroscopy. *Caution!* ^{99}Tc is a low-energy (0.292 MeV) β^- emitter with a half-life of 2.12×10^5 years. All manipulations of solutions and solids were performed in a laboratory approved for the handling of radioisotopes, and normal safety procedures were followed at all times to prevent contamination. Mass spectrum (electrospray): $m/z = 1043$ (MH^+, $[C_{38}H_{66}N_{13}O_{13}S_199Tc_1]$). IR (KBr disk): 976 cm^{-1} ($\gamma_{Tc=O}$).

Synthesis of the ^{99m}Tc Oxo Complex of RP128 ([^{99m}TcO(RP128)]). RP128 (200 μg, 0.2 μmol) was dissolved in 200 μL of saline. Sodium pertechnetate Tc-99m solution (10 mCi) was added to the solution, followed by tin(II) chloride (7.51×10^3 μg, 39 μmol) and sodium gluconate (1.3×10^3 μg, 5.8 μmol) in 100 μL of saline. The total volume of the solution was 340 μL. The solution was left at room temperature for 1 h. The ^{99m}Tc RP128 complex was analyzed by HPLC using method C. A radiochemical yield of greater than 85% was usually obtained. The amount of ^{99m}Tc microcolloid were determined by a TLC system developed for the clinical radiolabeling kit.[16] The TLC system measured the amount of ^{99m}Tc microcolloid and ^{99m}Tc pertechnetate. The radiochemical yield was higher than 90% as determined by the TLC system. The Re and ^{99m}Tc complexes of RP128 were co-injected into the HPLC. HPLC retention time using method C: ReO(RP128) $t_R = 13.99$, 14.27 min (UV detector set at 215 nm); ^{99m}TcO(RP128) $t_R = 14.62$ min (radiometric γ detector). HPLC retention time using method D: ReO(RP128) $t_R = 18.26$, 18.85 min (UV detector set at 215 nm); ^{99m}TcO(RP128) $t_R = 18.33$, 19.09 min (radiometric γ detector).

Tuftsin Receptor-Binding Assay. The protocol for the receptor-binding assay was adapted from a previously reported procedure.[25] HL-60 cells (ATCC #CCL-240) were grown in RPMI medium 1640 supplemented with 20% fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate and penicillin/streptomycin at 37 °C and 5% CO_2. Cells were harvested as required and washed with sucrose phosphate buffer (SPB: 250 mM sucrose and 50 μM phosphate, pH 7.4) and then resuspended in SPB. Cell viability and cell count were performed using trypan blue. Only cell suspensions of high viability (>80%) were used for binding, and these were suspended in SPB at a concentration of 10×10^6 viable cells per mL.

HL-60 cells, suspended in SPB, were used for saturation and competition assays. To determine the binding of ^{3}H TKPR, various concentrations (10−300 nM) were added to the cells. Nonspecific binding was determined by the addition of 10^{-4} M TKPPR. Competition

Scheme 1



assays were performed by co-incubating the HL-60s with ^{3}H TKPR (10^{-7} M) and a competing compound (TKPPR or Re oxo complex of RP128) at the desired concentration (10^{-12}−10^{-3} M). A duplicate set of all samples, but with no cells, was incubated to determine the nonspecific binding of ^{3}H TKPR to the glass-fiber GF/B filters (Whatman, 1 μm). All samples were incubated at 4 °C to prevent the internalization of the ligand−receptor complex. Incubation was terminated 45 min later by transferring the samples to the Millipore manifold filtration system, also at 4 °C. Cells were trapped by the glass-fiber filter disks and were then washed 3 times with 1 mL cold SPB. The filters were allowed to suction dry, collected and counted for remaining radioactivity using Beckman liquid scintillation β-counter. The counts from samples with no cells were subtracted from the duplicate set containing cells to calculate the cell bound cpm for each condition. This process negated the nonspecific binding to the filters.

The specific binding curve for ^{3}H TKPR was determined by subtracting the nonspecific binding from the total binding to the HL-60 cells. The dissociation constant (K_d) and number of receptors per cell were determined by the ^{3}H TKPR saturation analysis using GraphPad Prism Software. IC_{50} values for the individual test articles were calculated from the dose-dependent competition assays, also by GraphPad Prism. Values are represented as mean of 2−6 individual experiments ± SEM. Statistical significance ($p < 0.05$) was determined by students' *t*-tests using GraphPad Instat.

Results and Discussion

Dimethylglycyl-L-seryl-L-cysteinyl(acm)-glycyl-L-threonyl-L-lysyl-L-prolyl-L-prolyl-L-arginine (RP128) was prepared in excellent yields using solid-phase methods on an automated peptide synthesizer. RP128 was purified by HPLC and was characterized using NMR and electrospray mass spectrometry. ^{1}H and ^{13}C NMR spectral data of RP128 are given in Table 1.

The ^{1}H and ^{13}C NMR spectra of threonyl-L-lysyl-L-prolyl-L-prolyl-L-arginine (TKPPR) in D_2O and DSMO-d_6 have been previously reported.[29] The ^{13}C NMR resonances of the TKPPR portion of RP128 were very similar to the spectrum reported previously. Though the precise chemical shift values of the ^{1}H NMR resonances were not reported, a comparison of the ^{1}H spectrum for the TKPPR portion of RP128 to the reported ^{1}H NMR spectrum of TKPPR showed only minor differences. The nearly identical NMR spectral data between the TKPPR segment of RP128 and free TKPPR indicated that the attachment of the dimethylglycyl-L-seryl-L-cysteinyl-glycine chelator-linker did not significantly alter the conformation of the TKPPR sequence. There were differences between the reported[29] and observed chemical shifts for the α and β ^{13}C resonances on threonine. The minor differences in the NMR data were attributed to the differences in the pH at which the spectra were recorded; free TKPPR was recorded at a pH of 8.6,[29] while the RP128 NMR data were recorded at acidic pH. Changes to the chemical shift values for the threonine ^{13}C resonances with changes in pH was previously reported.[29]

Macroscopic studies of Tc coordination chemistry are performed using the low energy β-emitting ^{99}Tc radionuclide, which has a much longer half-life ($t_{1/2} = 2.12 \times 10^5$ years) than ^{99m}Tc ($t_{1/2}$ = 6.01 h). The ^{99}Tc oxo complex of RP128, [^{99}TcO-(RP128)], was prepared from the reduction of pertechnetate with $SnCl_2$ in the presence of deprotected RP128 (Scheme 1).

Coordination of deprotected RP128 to TcO^{3+} resulted in a more complex ^{1}H NMR spectrum. In the ^{1}H NMR spectrum of

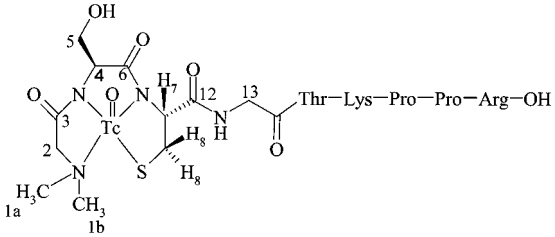
(29) Blumenstein, M.; Porter, P. L.; Najjar, V. A. *Biochemistry* **1979**, *18*, 5247.

RP128, the two methyl groups were chemically equivalent and a singlet at 2.834 ppm was observed. Upon complexation, the two methyl groups became nonequivalent and 2 singlets were observed at 2.376 and 3.444 ppm. In addition, the singlet at 3.972 ppm corresponding to the two methylene ^{1}H atoms in the dimethylglycine residue become diasterotopic, and two singlets were then observed at 4.6 and 4.129 ppm. These changes in the ^{1}H NMR resonances from the free RP128 to [^{99}TcO-(RP128)] indicate the coordination of the dimethylglycine N_{amine} atom to the Tc center. Changes to the serine and cysteine ^{1}H NMR resonances from the uncoordinated RP128 to the Tc complex were also observed. The resonances of the serine and cysteine methine ^{1}H atoms (H4, H7) shifted downfield approximately 0.35 and 1.1 ppm, respectively. The resonances of the nonequivalent methylene ^{1}H atoms on the cysteine side chain (H-8A and H-8B) underwent a downfield shift of approximately 0.8−0.9 ppm. The downfield shift of the ^{1}H NMR resonances clearly points to the coordination of the serine and cysteine N_{amide} atoms, and the cysteine $S_{thiolate}$ atom to the TcO^{3+} core. The coordination of RP128 to TcO^{3+} via the N_{amine} atom of dimethylglycine, the N_{amide} atoms of serine and cysteine, and the $S_{thiolate}$ atom of cysteine is consistent with what is observed in the Tc and Re oxo complexes of dimethylglycyl-L-seryl-L-cysteinyl-glycinamide (RP294).[30]

Like many Tc and Re oxo complexes with a $N_{4-x}S_x$ coordination moiety,[30−39] [TcO(RP128)] exists as two isomers; the serine CH_2OH side chain was in the syn and anti conformations with respect to the Tc oxo bond. The presence of the two isomers was evident from the NMR spectral data. In the ^{1}H NMR spectrum, there were two pairs of singlets associated with the two nonequivalent methyl groups in the dimethylglycine residue. Each pair of singlets corresponded to either the syn or anti [^{99}TcO(RP128)] isomer. In addition, there were two sets of ^{1}H NMR resonances for the methine and methylene hydrogen atoms of the dimethylglycine, serine, cysteine, and glycine residues. Table 2 summarizes the ^{1}H assignments for the glycine linker and the tripeptidic chelator portion of the [TcO(RP128)] complex for both the syn and anti isomers.

A comparison of the ^{1}H NMR spectral data for the ^{99}Tc complex of RP294[30] and the same 4 amino acids sequence in the [^{99}TcO(RP128)] complex showed only minor differences in chemical shifts and coupling constants. As in the Re and Tc RP294 complexes, the serine $CHCH_2OH$ side chain in [TcO-(RP128)] complex also adopted a strong preference for a single conformation. The two coupling constants, J_{AX} and J_{BX}, of the ABX spin system ($CH_XCH_AH_BOH$) were remarkably small in both the syn and anti isomers (syn J_{AX} = 2.9 Hz, J_{BX} = 2.4 Hz; anti J_{AX} = 3.1 Hz, J_{BX} = 1.9 Hz) and such small vicinal couplings show the CH_X proton to be preferentially gauche to both H_A and H_B. That is the OH group of the serine has a strong preference for the conformation where it is pointing in toward the Tc atom in both isomers.

Table 2. ^{1}H NMR Spectral Data for the Tc Oxo Dimethylglycyl-L-seryl-L-cysteinyl-L-glycine Segment of the [^{99}TcO(RP128)] Complex



assignment	δ (ppm)	J (Hz)	assignment	δ (ppm)	J (Hz)
H-7	5.694	$^3J_{AX}$ = 5.0	H-7	5.307	$^3J_{AX}$ = 1.2
		$^3J_{BX}$ = 6.6			$^3J_{BX}$ = 7.7
H-4	4.794	$^3J_{AX}$ = 3.0	H-4	4.600[a]	$^3J_{AX}$ = 2.9
		$^3J_{BX}$ = 1.8			$^3J_{BX}$ = 2.4
H-5A	4.243	$^3J_{AX}$ = 3.1	H-5A	4.130	$^3J_{AX}$ = 2.9
		$^2J_{AB}$ = 11.9			$^2J_{AB}$ = 11.9
H-5B	3.880	$^3J_{BX}$ = 1.9	H-5B	3.962	$^3J_{BX}$ = 2.4
					$^2J_{AB}$ = 11.9
H-8A[b]	3.757	$^3J_{AX}$ = 5.0	H-8A	3.874	$^3J_{AX}$ = 1.2
					$^2J_{AB}$ = 12.9
H-8B[b]	3.757	$^3J_{AX}$ = 6.6	H-8B	3.783	$^3J_{AX}$ = 7.8
					$^2J_{AB}$ = 13.0
H-2A	4.600*	$^2J_{AB}$ = 16.1	H-2A	4.145	$^2J_{AB}$ = 12.0
H-2B	4.129	$^2J_{AB}$ = 16.1	H-2B	3.994	$^2J_{AB}$ = 12.0
H-1A	2.376		H-1A	2.448	
H-1B	3.444		H-1B	3.455	
H-13A	3.834	$^2J_{AB}$ = 17.0	H-13A	3.834	$^2J_{AB}$ = 17.0
H-13B	3.765	$^2J_{AB}$ = 17.0	H-13B	3.765	$^2J_{AB}$ = 17.0

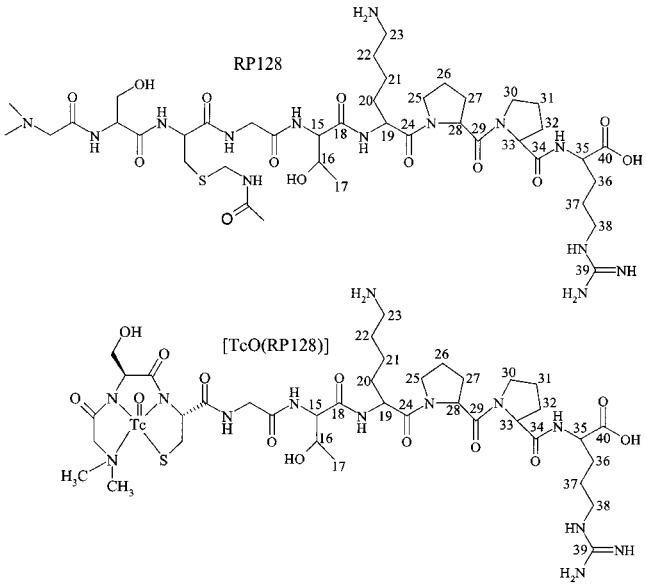
[a] Peaks hidden under water. [b] Selective TOCSY was unable to give $^2J_{AB}$.

An examination of the ^{1}H NMR spectral data for the TKPPR portion of the [TcO(RP128)] complex showed only minor differences in chemical shifts values compared to the same peptide sequence in uncoordinated RP128. Table 3 summarizes the chemical shifts of the ^{1}H resonances in the TKPPR segment of RP128 and [^{99}TcO(RP128)]. The nearly identical NMR spectral data showed that the coordination of RP128 to the TcO^{3+} core did not affect the three-dimensional structure of the TKPPR targeting moiety. A comparison of the same spectral data with that of previously reported data for free TKPPR also revealed only minor differences. This indicated that the technetium oxo chelate and the glycine linker did not significantly alter the conformation of the TKPPR sequence.

The Re(V) oxo complex of RP128 [ReO(RP128)] was prepared from the ligand exchange reactions of deprotected RP128 with $[ReO_2(en)_2]Cl$ (Scheme 1), where en is ethylenediamine. [ReO(RP128)] was purified by HPLC. It is well-known that Re(V) oxo complexes exhibit a characteristic $\upsilon_{Re=O}$ band in the infrared spectra. $ReOCl_3(PPh_3)_2$ has a $\upsilon_{Re=O}$ band at ~970 cm^{-1}, $[ReO_2(en)_2]Cl$ has a $\upsilon_{O=Re=O}$ band at ~820 cm^{-1}, and [ReO(RP294)] showed an $\upsilon_{Re=O}$ stretch at 986 cm^{-1}.[26,27,30,40,41]

(30) Wong, E.; Fauconnier, T.; Bennett, S.; Valliant, J.; Nguyen, T.; Lau, F.; Lu, L. F. L.; Pollark, A.; Bell, R. A.; Thornback, J. R. *Inorg. Chem.* **1997**, *36*, 5799.
(31) Fritzberg, A. R.; Klingensmith, W. C., III; Whitney, W. P.; Kuni, C. C. *J. Nucl. Med.* **1981**, *22*, 258.
(32) Kasina, S.; Fritzberg, A. R.; Johnson, D. L.; Eshima, D. *J. Med. Chem.* **1986**, *29*, 1933.
(33) Fritzberg, A. R.; Kasina, S.; Eshima, D.; Johnson, D. L.; Jones, A. G.; Lister-James, J.; Davison, A.; Brodack, J. W. *J. Nucl. Med*. **1984**, *25*, 16.
(34) Jones, A. G.; Davison, A. In *Technetium in Chemistry and Nuclear Medicine*; Deutsch, E., Nicolini, M., Wagner, H. N., Jr., Eds.; Cortina International: Verona, 1983; p 25.
(35) Edwards, D.; Cheesman, E.; Watson, M.; Maheu, L.; Nguygen, S.; Dimitre, L.; Nason, T.; Watson, A.; Walovitch, R. In *Technetium in Chemistry and Nuclear Medicine-3*; Nicolini, M., Bandoli, G., Mazzi, U., Eds.; Raven Press: New York, 1990; p 433.
(36) Bryson, N.; Dewan, J.; Lister-James, J.; Jones, A.; Davison, A. *Inorg. Chem.* **1988**, *27*, 2154.
(37) Rao, T.; Adhikesavalu, D.; Camerman, A.; Fritzberg, A. R. *J. Am. Chem. Soc.* **1990**, *112*, 5498.
(38) Kung, H. F.; Guo, Y.-Z.; Yu, C.-C.; Billings, J.; Subramanyam, V.; Calabrese, J. C. *J. Med. Chem.* **1989**, *32*, 433.
(39) Francesconi, L. C.; Graczyk, G.; Wehrli, S.; Shaikh, S. N.; McClinton, D.; Liu, S.; Zubieta, J.; Kung, H. F. *Inorg. Chem.* **1993**, *32*, 3114.
(40) Johnson, N. P.; Lock, C. J. L.; Wilkinson, G. *J. Chem. Soc.* **1964**, 1054.
(41) Chatt, J.; Rowe, G. A. *J. Chem. Soc.* **1962**, 4019.

Table 3. ^{1}H NMR Spectral Data for the TKPPR Portion of RP128 and [^{99}TcO(RP128)] Complex



^{1}H assignments	RP128 δ (ppm)	^{99}TcO(RP128) δ (ppm)
H-28	4.600	4.600
H-19	4.517	4.519
H-33	4.327	4.302
H-35	4.235−4.206	4.209
H-15	4.235−4.206	4.148
H-16	4.055	4.034
H-25 or H-30	3.732	3.582
H-25 or H-30	3.524	3.515
H-38	3.097	3.078
H-23	2.859	2.866
H-32 or H-27	2.228	2.253
H-32 or H-27	2.179	2.161
H-31 or H-26	1.907	1.931
H-31 or H-26	1.907	1.855
H-36 or H-20	1.818	1.702
H-37 or H-22	1.557	1.585
H-21	1.348	1.466
H-17	1.060	1.047

The infrared spectrum of [ReO(RP128)] showed an $\upsilon_{Re=O}$ stretch at 980 cm^{-1}. The mass spectrum of [ReO(RP128)] was obtained by electrospray mass spectrometry in the positive ion detection mode. A MH^{+} ion was detected. The mass spectral and elemental analysis data confirmed the ReO(RP128) complex as a mononuclear monoligand complex. The coordination of ReO^{3+} with the chelator-linker segment of RP128 was not expected to differ from that of the Tc analogue. No decomposition of [ReO(RP128)] was observed when the compound was placed in aqueous solution at neutral pH for 24 h.

The ^{99m}Tc complex of RP128 was prepared at the tracer level via the reduction of pertechnetate in the presence of excess tin(II) chloride and sodium gluconate (Scheme 1). The ^{99m}Tc and Re complexes of RP128 were co-injected into the HPLC. The two complexes coeluted from the HPLC. Both the syn and anti isomers of the Tc and Re oxo complexes were observed on the HPLC chromatogram**.** The nearly identical retention times of the Re and ^{99m}Tc complexes support the hypothesis that the Re complex has a similar structure to that of the Tc complex.

The peptide threonyl-L-lysyl-L-prolyl-L-prolyl-L-arginine (TKPPR) and the rhenium complex of RP128, [ReO(RP128)] were tested for tuftsin receptor binding affinities using HL-60 cells. Saturation dose-dependent binding of tritated TKPR to HL-60 cells was observed, with a K_d value of 1.4×10^{-7} M and B_{max} of 6.8×10^{-11}. It was determined that there were approximately 20.5×10^{6} tuftsin receptors per HL-60 cell. The calculated K_d value for TKPR was greater than the previously reported value of 4.7×10^{-8} M.[25] Both TKPPR and the Re RP128 complex inhibited the binding of TKPR to HL-60 cells in a concentration dependent manner. The average IC_{50} value for TKPPR was 2.3 $\pm$ 0.8 pM. The IC_{50} value for the [ReO(RP128)] was 5.0 $\pm$ 0.2 pM. The similar binding affinities between TKPPR and [ReO(RP128)] to the tuftsin receptor suggested that the rhenium oxo dimethylglycyl-L-seryl-L-cysteinyl-glycine segment did not significantly affect the binding affinity of TKPPR to the tuftsin receptor. The results of the receptor binding assay were consistent with the findings of the NMR studies.

Conclusion

The Tc(V) and Re(V) oxo complex of dimethylglycyl-L-seryl-L-cystyl-glycyl-L-threonyl-L-lysyl-L-prolyl-L-prolyl-L-arginine (RP128) were prepared successfully. NMR spectral data showed that the dimethylglycyl-L-seryl-L-cystyl-L-glycine segment of RP128 coordinated to TcO^{3+} and ReO^{3+} as expected, via the N_{amine} of dimethylglycine, N_{amide} of serine and cysteine, and the $S_{thiolate}$ of cysteine. Both the syn and anti isomers were observed in the Re and Tc complexes. The NMR spectral data of RP128 and [TcO(RP128)] suggest that the attachment of dimethylglycyl-L-seryl-L-cystyl-glycine and the coordination of the metal oxo core (MO^{3+}) via the tripeptidc chelator did not significantly altered the conformation of the TKPPR tuftsin receptor targeting sequence. This was supported by the preliminary receptor binding data, which showed that [ReO(RP128)] had a similar binding affinity to TKPPR. The ^{99m}Tc(V) oxo complex of RP128 represents a tuftsin receptor targeting agent.

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