Isolation, Characterization, and Biological Evaluation of *Syn* and *Anti* Diastereomers of [^{99m}Tc]Technetium Depreotide: a Somatostatin Receptor Binding Tumor Imaging Agent

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The early and later eluting [^{99m}TcO]depreotide products on RP-HPLC were confirmed to be the *anti* and *syn* diastereomers, respectively, based on proton NMR and circular dichroism spectroscopy. NMR provided evidence of a folded, conformationally constrained structure for the *syn* diastereomer. The *syn* diastereomer is predominant (*anti*/*syn* ∼ 10:90) in the [^{99m}TcO]depreotide preparation and shows a slightly higher affinity (IC_{50} = 0.15 nM) for the somatostatin receptor than the *anti* diastereomer (IC_{50} = 0.89 nM). Both diastereomers showed higher binding affinities than the free peptide (IC_{50} = 7.4 nM). Biodistribution studies in AR42J tumor xenograft nude mice also showed higher tumor uptake for *syn* [^{99m}TcO]depreotide (6.58% ID/g) than for the *anti* [^{99m}TcO]depreotide (3.38% ID/g). Despite the differences in biological efficacy, the favorable binding affinity, tumor uptake, and tumor-to-background ratio results for both diastereomeric species predict that both are effective for imaging somatostatin receptor-positive tumors in vivo.

Introduction

The observation that tumors express somatostatin receptors (SSTR[a]) in greater density than in normal tissues[1] has generated a great deal of interest in radiolabeled somatostatin analogs for tumor imaging.[2−6] The first peptide-based receptor binding radiopharmaceutical was ^{111}In-DTPA-octreotide.[3,7] Octreotide (D-phenylalanyl-1-cysteinyl-1-phenylalaninyl-D-tryptophyl-1-lysyl-1-threonyl-*n*-(2-hydroxy-1-(hydroxymethyl)propyl)-1-cysteinamide cyclic (2−7)-disulfide) is a shortened peptide analog of somatostatin, which is a cyclic disulfide-containing peptide hormone of 14 amino acids that is present throughout the central nervous system.

The success of ^{111}In DTPA-octreotide stimulated the search for receptor-specific imaging agents based on ^{99m}Tc peptides. A somatostatin derivative radiolabeled with ^{99m}Tc would offer advantages over an ^{111}In product, because the ^{99m}Tc radionuclide has a more favorable photon abundance for gamma imaging, a more convenient half-life (6 h vs 60 h), and is more readily available.

Depreotide (cyclo-[(*N*-Me)Phe-Tyr-D-Trp-Lys-Val-Hcy]CH_2-CO.β-Dap-Lys-Cys-Lys.amide, P829) is a SSTR binding peptide that has been developed as a tumor-imaging radiopharmaceutical due to its optimal biodistribution, in vivo half-life, and somatostain receptor (SSTR) binding affinity.[8] The ^{99m}Tc-labeled agent, [^{99m}TcO]depreotide, has received regulatory approval in the United States and Europe for use in the detection of malignant lung cancer. [^{99m}TcO]depreotide binds to a wider range of somatostain receptor subtypes (SSTR2, SSTR3, and SSTR5)[9] than octreotide and its radiolabeled derivatives (mainly these bind to SSTR2[10,11]) and, therefore, may be useful for diagnosing a broader range of tumor types. [^{99m}TcO]depreotide has shown promise in the detection of a variety of tumors, including lung cancer,[12] lymphoma,[13] breast cancer,[14] melanoma,[15] and colorectal carcinoma.[16]

[TcO]Depreotide, Figure 1, is comprised of a cyclic hexapeptide containing a SSTR binding sequence and a linear tetrapeptide that was designed to form a coordination complex with technetium at the Dap-Lys-Cys sequence (Dap = β-diaminopropioninc acid). The chelation about the technetium is denoted "N_3S" because the coordinating atoms in the peptide include three nitrogens (two amide nitrogen atoms of Lys and Cys and one amine nitrogen of Dap) and one sulfur (the thiol sulfur of Cys). Based on previous results for technetium(V) complexes of peptide-based ligands of the N_3S type,[17−20] the [TcO]-depreotide complex is expected to have a distorted square pyramidal structure, with the oxo group perpendicular to the plane of the peptide nitrogen and sulfur coordinating atoms.

Diasteromers of technetium (V) are known to form when ligands contain chiral centers on the portion of the chelate which constitutes the coordination plane. For such ligands, the technetium complex can form with the substituents either *syn* or *anti* to the Tc═O group.[21] Three chiral centers are incorporated into the coordination plane of [^{99m}TcO]depreotide, therefore, two distinct diastereomers are predicted, and these are shown in Figure 1. For discussion here, the two diastereomers will be referred to as *syn* or *anti* with regard to the position of the Lys^2 (the lysine which chelates to the technetium) side chain relative to the Tc═O bond. Two main products are indeed formed when the depreotide peptide is radiolabeled with ^{99m}Tc, and these are purported to be the *syn* and *anti* diastereomers of [^{99m}TcO]depreotide.

When a radiopharmaceutical product consists of two diastereomers, it is important to assign the two products (e.g., as either

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[a] Abbreviations: SSTR, somatostatin receptor; SSTR2, somatostatin receptor subtype 2; SSTR3, somatostatin receptor subtype 3; SSTR5, somatostatin receptor subtype 5; DTPA, diethylenetriaminepentaacetic acid; DOTA, 1,4,7,10-tetraazacyclododecanetetraacetic acid.

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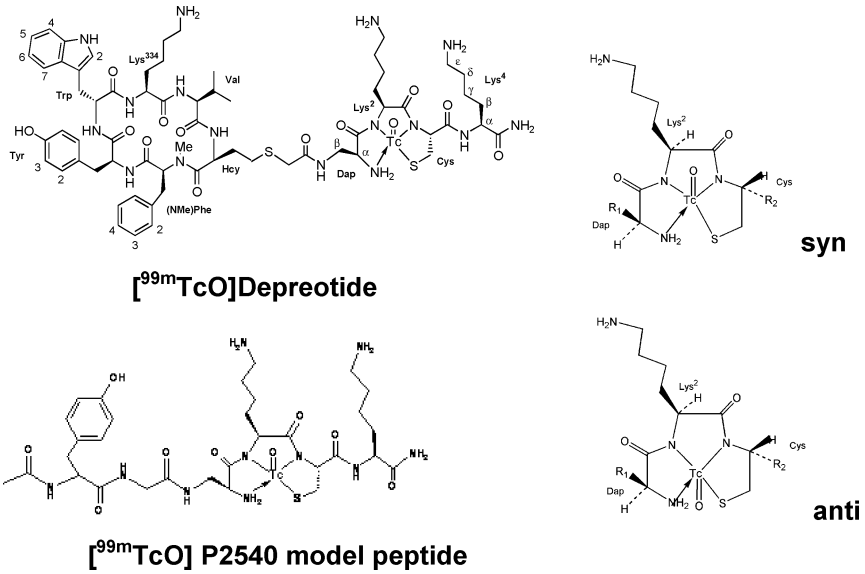

Figure 1. Top left: [TcO]depreotide. Sequence and labeling system used for NMR analysis. Bottom left: [TcO]depreotide model peptide. Right: technetium binding regions of [TcO]depreotide showing possible *syn* and *anti* diastereomers (*syn* and *anti* relative to the Lys side chain).

syn or *anti*) accurately for thorough characterization of the drug. It is well-established that stereoisomers of radiopharmaceutical products often have very different biodistribution behavior. In cases where radiopharmaceuticals can form two distinct isomeric species, it is important to evaluate the individual products separately to ensure that they both possess good biological efficacy.

Therefore, the overall goal of this project was (1) to unambiguously assign the *syn* and *anti* diastereomers of [^{99m}TcO]-depreotide and confirm the Dap-Lys-Cys Tc binding site in depreotide and (2) to evaluate the biological behavior of each diastereomer and the mixture. The first objective was achieved by synthesizing macroscopic quantities of each diastereomer of [^{99}TcO]depreotide for spectroscopic characterization. The unambiguous assignment of the *syn* and *anti* diastereomers was made possible by other studies of Tc complexes of tripeptide ligands similar to the [TcO]depreotide metal center, which will be reported elsewhere.[22] These studies consisted of crystallizing and structurally characterizing Tc tripeptide complexes and correlating their stereochemistry to characteristic NMR and circular dichroism (CD) spectroscopic features. A small model peptide, Ac-Tyr-Gly-β-Dap-Lys-Cys-Lys.amide (P2540; Figure 1), that possesses the exact chelator portion and the C-terminus of depreotide was also made and characterized in the work reported here to further confirm the [TcO]depreotide *syn* and *anti* diastereomer assignments made by the NMR and CD techniques.

The isolation of pure samples of the *syn* and *anti* diastereomers of [^{99}TcO] and [^{99m}TcO]depreotide products allowed for fulfillment of the second objective, that is, determination of the in vitro and in vivo biological properties of the diastereomers and correlation of structure with biologial activity. The isolation of the [TcO]depreotide complexes, their characterization, and their biological evaluation are the focus of this report.

Experimental Section

Symbols and abbreviations for amino acids generally followed IUPAC-IUB recomendations. Depreotide (P829 Peptide), cyclo-[(*N*-Me)Phe-Tyr-D-Trp-Lys-Val-Hcy]CH_2CO.β-Dap-Lys-Cys-Lys.amide, was synthesized using Fmoc solid-phase peptide synthesis (SPPS), as described previously.[8] NMR chemical shift data for depreotide are given in Table S1. P2540 peptide, Ac-Tyr-Gly-β-Dap-Lys-Cys-Lys.amide, was also synthesized using standard Fmoc SPPS techniques, purified by reversed phase HPLC, and lyophilized. The final product had purity >96% and had the expected molecular mass by MS analysis ([M + H$^+$] expected = 725.0; found = 725.39). Octreotide was also prepared using standard SPPS procedures. [$N(C_4H_9)_4$] [^{99}TcOCl_4] was prepared according to a literature preparation.[23] Na[^{99}TcO(ethylene glycol)$_2$] or (Na[^{99}TcO$(eg)_2$]) was prepared from [^{99}TcOCl_4]$^-$ by published methods.[24] (3-^{125}I-iodotyrosyl^{11})somatostatin-14 (tyr^{11}) or ^{125}I-somatostatin was obtained from Amersham Corp. and used as received. Lyophilized kits for the preparation of technetium Tc 99m depreotide injection (NeoTect), containing 50 μg of depreotide, 5 mg of sodium α-D-glucoheptonate dihydrate, 100 μg of edetate disodium USP (EDTA), and 50 μg of tin(II) chloride dihydrate formulated at pH 7.4 were provided by Diatide, Inc. Sodium pertechnetate Tc 99m injection was obtained from a ^{99}Mo/^{99m}Tc generator on the day of use. Methanol and acetonitrile (Fisher Scientific) were HPLC grade. All other chemicals and solvents were reagent grade and used as received. ^{99}Tc is a weak β-emitter (0.29 keV, half-life 2.12×10^5 y); all reactions with ^{99}Tc were carried out in laboratories approved for use of radioactivity. ^{99}Tc was purchased as NH_4TcO_4 from Oak Ridge National Laboratory. A small sample was added to water and treated with H_2O_2 to oxidize any TcO_2; the resulting clear solution was standardized using UV spectroscopy.[25]

Analytical HPLC was performed on a Waters or Agilent system using a 250 mm Zorbax 300SB C18 column (Diatide or Schering AG) or on a Rainin Dynamax system using a 3.9×150 mm Waters Delta-Pak C18 column (Hunter College) with 0.1% (v/v) trifluoroacetic acid (TFA) mobile phases in acetonitrile/water (mobile phase A = 0.1% TFA in H_2O; mobile phase B = 0.1% TFA in 90/10 acetonitrile/water) and flow rates at 1.0 or 1.2 mL/min. Preparative HPLC of the [^{99}TcO]depreotide isomers was performed under similar conditions on the Rainin system with a Delta-Pak C18 preparative HPLC column (19×300 mm). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were collected on a Finnigan MAT Vision 2000 mass spectrometer with a nitrogen laser at 337 nm. Infrared spectra were recorded on a Perkin-Elmer FT-IR spectrometer. ^{1}H NMR spectra were obtained on a Varian Unity Plus 500 MHz spectrometer. The following two-dimensional experiments were performed at 20 °C: double-quantum filtered correlation spectroscopy (DQF COSY), using a presaturation water suppression method; total correlation spectroscopy (TOCSY) with a mixing time of 60 ms using a combined pulsed field gradient (PFG); WATERGATE water suppression method; and rotating framed nuclear Overhauser enhancement spectroscopy (ROESY), with a mixing time of 200

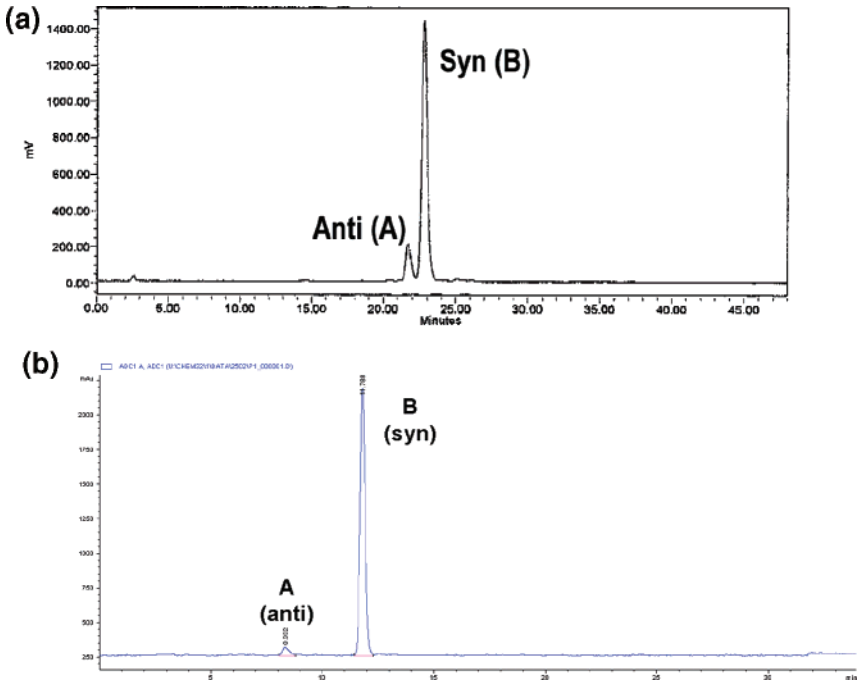

Figure 2. HPLC chromatogram (radiometric detection) of radiolabeled product (a) [^{99m}TcO]depreotide and (b) [^{99m}TcO]P2540. Product A (*anti* diastereomer) and product B (*syn* diastereomer) are noted (conditions: Zorbax 300SB C18 column; mobile phases 0.1% v/v TFA in acetonitrile/water; gradients, (a) 24−27% B over 25 min, then 27−68% B over 17 min; flow rate 1.2 mL/min; (b) 10−14% B over 20 min; flow rate 1.0 mL/min).

ms, using a presaturation water suppression method. The DQF COSY spectrum was recorded as 256 × 4096 complex points and subsequently processed to yield a 1024 × 4096 data matrix. The TOCSY spectrum was recorded as 512 × 4096 complex points and processed to yield a 1024 × 4096 matrix. The ROESY spectrum was recorded as 512 × 4096 complex points and processed to give a 1024 × 4096 data matrix. One-dimensional spectra were also taken. NMR samples were dissolved in degassed aqueous 0.01 M HCl (to ensure detection of amide protons) with added D_2O for use as a deuterium lock by the NMR spectrometer.

Circular dichroism spectra of methanolic solutions of the diastereomers of [^{99}TcO]depreotide and [^{99}TcO]P2540 tetrapeptide were collected on a JASCO-J710 spectropolarimeter, where the optical system was fed by a prepurified nitrogen compressed gas at a flow rate of 5 L/min. A total of 1 mg of ^{99}Tc depreotide or tetrapeptide diastereomer complex was dissolved in 1 mL of methanol. A total of 250 μL of the aliquot was placed in a 250 μL CD cell. The sample was scanned from 170 to 600 nm with ±50 degree sensitivity. The CD spectra were collected with baseline and background corrections using pure methanol.

Preparation of Radiolabeled Product [^{99m}TcO]depreotide from Kit for the Preparation of Technetium Tc 99m Depreotide Injection and Isolation of *Syn* and *Anti* [^{99m}TcO]depreotide. In a standard preparation of [^{99m}TcO]depreotide, a vial of the Kit for the Preparation of Technetium Tc 99m Depreotide Injection was reconstituted with 10−50 mCi of sodium pertechnetate Tc 99m injection and normal saline totaling approximately 1.0 mL. The vial was heated in a boiling water bath for 10 min and then allowed to cool for 15 min at room temperature. The radiochemical purity was typically >90% by reversed phase HPLC (conditions: 24−27% B TFA over 25 min, then 27−68% B TFA over 17 min). A typical HPLC chromatogram showing the radiolabeled product profile is given in Figure 2a. The radiolabeled A/B product ratio could be adjusted to produce more of product A by adding additional stannous tin (0.2 mL of saturated aqueous stannous tartrate) to the preparation and by incubating at a lower temperature (25−60 °C). The radiolabeled ^{99m}Tc material thus prepared coeluted with the isolated [^{99}TcO]depreotide *syn*/*anti* isomers on HPLC (Supporting Information, Figure S1).

The individual diastereomers (products A and B) of [^{99m}TcO]-depreotide were isolated from a 200 μL (10 mCi) injection of the radiolabeled product on reversed phase HPLC using the standard analytical HPLC method described above. Product A (R_t = 24.1 min) and product B (R_t = 25.6 min) were collected in approximately 2 min fractions. The two fractions were treated with a slow sparge of nitrogen for 20 min to remove acetonitrile from the samples. After removal of acetonitrile, 50 μL of 0.2 M phosphate buffer pH 7.4 was added to a 500 μL portion (~600 μCi) of each isomeric sample fraction to yield samples with pH 7−7.5. To account for nonradioactive components normally found in preparations of NeoTect, a separate depreotide kit vial was reconstituted in 1.0 mL of saline, and 20 μL of the cold reconstituted kit was added to the isolated A/B samples for each mCi of HPLC-purified material. Isolated fractions of products A/B [^{99m}TcO]depreotide had ≥80% radiochemical purity by HPLC with ≥90% isomeric purity.

[^{99m}TcO]P2540 was prepared using a similar radiolabeling procedure. "Placebo" kits were prepared that were equivalent to the depreotide kits, except that they contained no depreotide peptide. Hence, these placebo kits contained 5 mg sodium α-D-glucoheptonate dihydrate, 100 μg edetate disodium (EDTA), and 50 μg tin-(II) chloride dihydrate at pH 7.4 lyophilized in a 5 mL vial. P2540 peptide was dissolved in normal saline at 1 mg/mL, and 50 μg (50 μL) of peptide was added to the placebo kit. Vials were reconstituted with ^{99m}Tc pertechnetate generator eluate + saline totaling 1.0 mL and containing ~20 mCi, heated for 10 min in a boiling water bath, and cooled at room temperature for ~ 10 min. A typical HPLC chromatogram for [^{99m}TcO]P2540 is shown in Figure 2b.

Synthesis of [^{99}TcO]Depreotide Product A (*Anti* Diastereomer). Depreotide trifluoroacetate (30.8 mg of depreotide, 0.0158 mmol, corrected for peptide content) was dissolved in 0.8 mL of 0.1 M sodium acetate buffer, pH 3.5. A sample of $Na[TcO(eg)_2]$ (4.3 mg, 0.0167 mmol) was dissolved in approximately 0.2 mL of methanol and added to the depreotide solution with stirring. The reaction mixture immediately changed to dark brown. After stirring for 1 h, analytical HPLC (conditions: 43% B isocratic) showed unreacted peptide and products A and B, with an A/B ratio of approximately 2:1.

The product was purified by preparative HPLC in two injections (0.5 mL each) using isocratic 43% B HPLC conditions. The first fraction to elute from the HPLC was collected. Fractions were collected based on effluent monitoring at 220 nm. The first of the

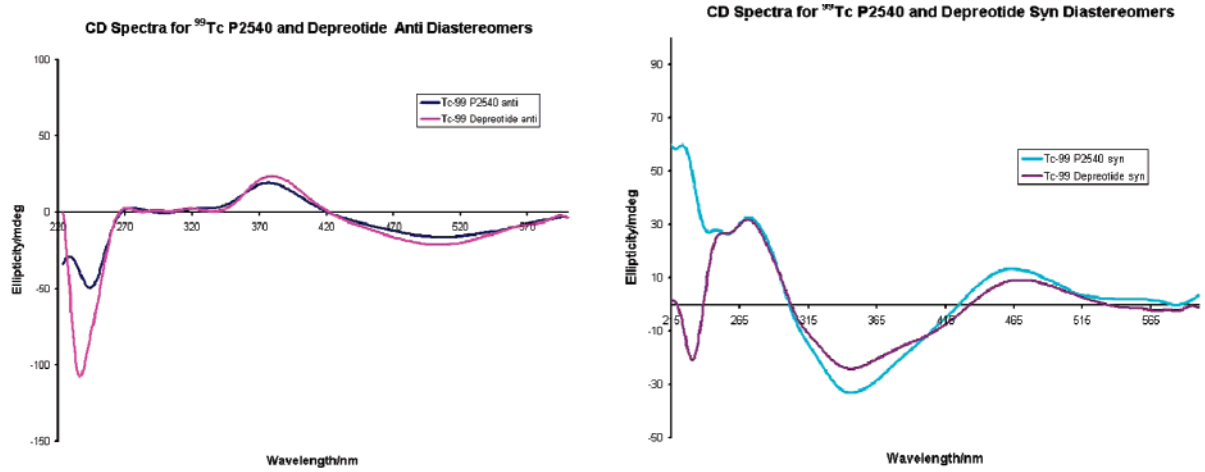

Figure 3. Circular dichroism spectra for [^{99}TcO]depreotide and [^{99}TcO]P2540. Left, products A, *anti* diastereomers; and right, products B, *syn* diastereomers.

four fractions from each run proved to contain suitably pure (>95%) product A. These fractions were combined and lyophilized to yield 4 mg (0.0029 mmol; 19%) of pink solid. Analytical HPLC of [^{99}TcO]depreotide product A (Figure S1) indicated that the sample was >95% pure. HPLC concordance experiments showed that [^{99}TcO]depreotide product A coeluted with ^{99m}Tc depreotide verifying that the structure and chemistry are identical (Supporting Information, Figure S1). MALDI TOF MS *m*/*z* (assignment): 1491.3 $(M + Na)^+$ (Figure S2). IR (KBr): (Tc═O stretch) 979 cm^{-1} (Figure S3). ^{1}H NMR (500 MHz, 0.1 M HCl/D_2O) is given in Table S2 and Figure S4. Figure 3 shows the CD spectum for [^{99}TcO]depreotide, product A.

Synthesis of [^{99}TcO]Depreotide Product B (*Syn* Diastereomer). Depreotide trifluoroacetate (60.5 mg of depreotide, 0.0316 mmol, corrected for peptide content) was dissolved in 2 mL of methanol. A stock solution of Na[TcO$(eg)_2$] was made by dissolving 14 mg in 2 mL of methanol. A 1.5 mL portion of the stock Na-[TcO$(eg)_2$] solution (0.0408 mmol) was added at room temperature to the peptide solution with stirring. The reaction mixture immediately turned brown. Analytical HPLC (conditions: 30−80% B TFA gradient over 45 min) of the reaction solution indicated the reaction was complete at 1 h.

The product was purified by preparative HPLC in three preparative runs, with injection volumes of 500, 500, and 300 μL. The main peak was collected and lyophilized for each run. The three lyophilized samples were redissolved in 0.1% TFA, combined, and repurified by preparative HPLC in two injections. Fractions were collected based on effluent monitoring at 220 nm. Two fractions of acceptable purity (>95%) were obtained, yielding two samples of yellow lyophilized product B material. Overall yield was 18 mg, (0.0132 mmol, 32%). Analytical HPLC of [^{99}TcO]depreotide product B (Figure S1) indicated that the sample was >95% pure. The [^{99}TcO]depreotide product B coeluted with the [^{99m}TcO]-depreotide product B, verifying that structure and chemistry are identical between the tracer and macroscopic complexes (Supporting Information, Figure S1). MALDI TOF MS *m*/*z* (assignment): 1469.5 $(M + H)^+$ (Supporting Information, Figure S2). IR (KBr): (Tc═O stretch) 979 cm^{-1} (Supporting Information, Figure S3). ^{1}H NMR (500 MHz, 0.1 M HCl/D_2O is given in Supporting Information, Table S3 and Figure S4. Figure 3 shows the CD spectum for [^{99}TcO]depreotide, product B.

Synthesis of [^{99}TcO]P2540 (*Syn* and *Anti* Diastereomers). In a microcentrifuge vial, 16.5 mg P2540 peptide was dissolved in 200 μL of ultrapure water. In another microcentrifuge vial, 10.36 mg $N(C_4H_9)_4$ $^{99}TcOCl_4$ was dissolved in 200 μL of methanol followed by the addition of 5 drops of ethylene glycol (eg) to form a clear, blue solution. The $[^{99}TcO(eg)_2]^-$ solution was then added to the water-based peptide dropwise. The solution turned yellow-orange instantly, and after several minutes, the solution turned dark-brown.

Analytical HPLC on the reaction solution showed two major peaks at 8.1 and 12.4 min (column, Waters DeltaPak 5μ C_{18} 100 Å, 3.9 × 150 mm; mobile phase ((A) 0.1% TFA in H_2O and (B) 0.1% TFA in acetonitrile/water (90:10); gradient, 4−8% B over 15 min, then 8−30% B over 2 min, followed by 4% B over 3 min; flow rate, 1.0 mL/min; software, Star Chromatography Workstation Version 6) with a total run time of 20 min. The ratio of the two products by HPLC was ~30:70. The two products were then purified by preparative HPLC using the following conditions: column, Waters DeltaPak 5μ C_{18} 300 Å, 19.0 × 300 mm; mobile phase, same as for analytical; gradient, 4−8% B over 30 min, followed by 8−30% B over 4 min, followed by 4% over 6 min; flow rate, 24.0 mL/min.

For the preparative run, a 200 μL of crude reaction mixture was injected and fractions were collected at 20 and 27 min with the total run time of 40 min. The two fractions of product A and three fractions product B were collected and lyophilized to yield solid samples of product A (yield = 19%) that were pink in color and product B (yield = 58%) that was yellow in color.

Analytical HPLC indicated that the isolated [^{99}TcO]P2540 products A and B were >95% pure (Supporting Information, Figure S5). ^{1}H NMR (500 MHz, 0.1M HCl/ D2O), see Supporting Information, Table S4 and Figure S6 for assignments. Electrospray MS−product A: *m*/*z* (assignment): 837.4 $(M + H)^+$, 419.3 $(M + 2H)^{2+}$; monoisotopic mass, 835.25; average mass, 836.76. Product B: *m*/*z* (assignment): 837.4 $(M + H)^+$, 419.3 $(M + 2H)^{2+}$; monoisotopic mass, 835.25; average mass, 836.76. Circular dichroism: see Figure 3 and Supporting Information, Figure S7.

Cell Membrane Somatostatin Receptor Binding Affinity Studies. Rat pancreatic AR42J cells (ATCC Rockville, MD) were cultured in modified Ham's F12K medium containing 20% fetal bovine serum in 95% humidity and 5% CO_2. Cells were harvested by centrifugation and implanted subcutaneously into female nude mice (6−8 weeks old). Three weeks after implantation, tumors were dissected, minced, and implanted again into nude mice. After three more weeks, the second-passage tumors were harvested for tumor membrane preparation. Tumors were dissected from mice, minced to a fine paste, and homogenized in 10 mM sodium bicarbonate at pH 7.8 containing 1 mM EDTA, 1 mM EGTA, 2.5 mM dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 0.5 μg/mL aprotinin, and 200 μg/mL bacitracin (homogenization buffer). Membranes were washed three times by centrifugation at 45 000 × *g* in 35 mL of homogenization buffer at 4 °C. The final resuspension was 200 μg membrane protein/mL in assay buffer.

The assay for *syn*/*anti* [^{99}TcO]depreotide was conducted with the isolated radiolabeled tracer *syn* or *anti* [^{99m}TcO]depreotide as the competing ligand. The assay (250 μL) consisted of tumor membranes (50 μg of membrane protein), 0.3 μCi of isolated *syn* or *anti* [^{99m}TcO]depreotide, and *syn* or *anti* ^{99}Tc isomer in assay buffer at 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 nM concentra-

tions. Assay buffer was 50 mM Tris-HCl at pH 7.8 containing 1 mM EDTA, 5 mM $MgCl_2$, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 0.5 μg/mL aprotinin, and 200 μg/mL bacitracin. The assay samples were incubated at room temperature for 30 min in a shaking water bath, and the incubation was terminated by filtration through polyethyleneimine pretreated GF/C glass fiber filters. All determinations are run in duplicate.

The assay for depreotide was conducted by an identical procedure as that described above for *syn*/*anti* [^{99}TcO]depreotide, except that ^{125}I-somatostatin (0.1 μCi) was used as the competing ligand, and the final concentrations of depreotide were 100 to 0.1 nM.

Biodistribution in a Mouse Tumor Xenograft Model. Rat pancreatic AR42J cells (ATCC Rockville, MD) were cultured, inoculated into nude mice, and then reinoculated as described above for cell binding studies. The mice possessing the second passage tumors were used in biodistribution studies. A dose of 10 to 50 μCi/mouse of [^{99m}TcO]depreotide clinical preparation (mixture) or HPLC-purified *syn* or *anti* [^{99m}TcO]depreotide isomer were injected intravenously within 30 min of preparation (clinical preparation) or the HPLC separation (isolated diastereomers). To test for blocking of tumor uptake by a known SSTR ligand, some animals were injected subcutaneously 200 μg/200 μL with octreotide 30 min prior to the injection of *syn* or *anti* isomer. In addition, 100 μg/100 μL of octreotide was coinjected with the *syn* or *anti* isomer at time zero. Control animals received 200 μL of saline. Mice were sacrificed 90 min postinjection by decapitation, and the trunk blood was collected. Tumors (1−3 per mouse) and samples of muscle from both legs (*quadriceps femoris*) were collected, weighed, and counted for radioactivity.

Results and Discussion

^{99m}Tc Radiolabeling of Depreotide. [^{99m}TcO]depreotide is typically prepared by addition of sodium pertechnetate Tc 99m injection and normal saline totaling approximately 1.0 mL to a vial of "Kit for the Preparation of Technetium Tc 99m Depreotide Injection (NeoTect)" followed by heating in a boiling water bath. Two radiolabeled products are observed by reversed phase HPLC in approximately a 10:90 ratio (first-eluting/second-eluting), as shown in Figure 2. The model compound [^{99m}TcO]P2540 also displayed two products in reversed phase HPLC (Figure 2b) with a similar ratio, favoring the second-eluting compound. The first-eluting products by reversed phase HPLC for [^{99m}TcO]depreotide and [^{99m}TcO]P2540 were designated product A, while the second eluting products were designated product B. These have been assigned to the *anti* and *syn* diastereomers, respectively, vide infra.

Synthesis and HPLC, MS, IR Characterization of [^{99}TcO]-Depreotide Isomeric Products A and B. To characterize [^{99m}TcO]depreotide, the long-lived isotope of technetium, ^{99}Tc ($t_{1/2} = 2.13 \times 10^5$ years), available in macroscopic quantities, is used. The reaction of TFA depreotide with Na[^{99}TcO(eg)$_2$] in methanol solution with no pH adjustment resulted in predominantly product B. With adjustment to pH 3.5, using acetate buffer, the product mixture favored product A (product A/product B, approximately 2/1). For P2540, reaction in methanol/water with Na[^{99}TcO(eg)$_2$] gave [^{99}TcO]P2540 product A/product B in ∼30/70 ratio. The individual diastereomeric species were isolated from these reaction solutions by reversed-phase preparative HPLC and lyophilization of appropriate fractions to give products of >95% purity. The isolated products A or B were also observed to coelute on HPLC (simultaneous UV and radiometric γ detection), with the [^{99m}TcO] radiolabeled products prepared separately (Figure S1). The colors of the two products were strikingly different: products A (*anti*) were pink, whereas products B (*syn*) were yellow; this is consistent with the color profiles for ^{99}Tc tripeptide species.[22]

[^{99}TcO]depreotide isolated products A and B were analyzed by positive-ion MALDI-TOF mass spectrometry. For product A, the most abundant peak ($m/z = 1491.3$) was assigned as a sodium adduct $M + Na^+$. The mass corresponding to a sodium adduct of a technetium complex of depreotide is predicted as (depreotide $- 3H^+ + Na^+ +$ TcO); $C_{65}H_{93}N_{16}O_{13}S_2TcNa$; $MW_{average} = 1491.7$. For product B, the observed mass of the most abundant peak ($m/z = 1469.5$) was assigned to $M + H^+$ (depreotide $- 3H^+ + H^+ +$ TcO); $C_{65}H_{94}N_{16}O_{13}S_2Tc$; $MW_{average} = 1469.7$. Within the error of the experiments, both analyses are consistent with the formulation shown in Figure 1, with the formula $C_{65}H_{93}N_{16}O_{13}S_2Tc$; $MW_{average} = 1469$.

The IR spectra for both [^{99}TcO]depreotide products A and B each contained a peak at 979 cm^{-1} attributed to the Tc═O stretch, which was not present in the spectrum of the peptide alone. This value for the Tc═O stretching frequency is in the expected range of 900−1000 cm^{-1} for technetium(V) oxo complexes[26−29] The equivalence in molecular masses and in the Tc═O stretching frequencies for products A and B is consistent with the assignment of the two products as diastereomers.

^{1}H NMR Characterization of [^{99}TcO]Depreotide *Syn* and *Anti* Diastereomers. Two-dimensional ^{1}H NMR studies, including double-quantum filtered correlation spectroscopy (DQF COSY), total correlation spectroscopy (TOCSY), and rotating framed nuclear Overhauser enhancement spectroscopy (ROESY) were conducted on depreotide and the isolated [^{99}TcO]depreotide products A and B. The COSY results identified all of the amino acids and structural features of the compounds, and the TOCSY and ROESY experiments confirmed the connectivities of the amino acids (thereby confirming the peptide sequences). The ROESY experiments also identified interactions between non-adjacent residues in the *syn* (product B) structure (vide infra). Positional assignments for the NMR analyses are shown in the structure given in Figure 1. Selected plots of the one-dimensional, COSY, ROESY, and TOCSY ^{1}H NMR data for the *syn* and *anti* [^{99}TcO]depreotide diastereomers are given in Figure S4, and chemical shift assignments for all protons in depreotide, [^{99}TcO]depreotide A (*anti*), and [^{99}TcO]depreotide B (*syn*) are given in Tables S1, S2, and S3, respectively. A similar complete tabulation of proton chemical shift assignments for P2540 peptide, [^{99}TcO]P2540 product A, and [^{99}TcO]P2540 product B is given in Table S4. NMR signals for Cys or Lys^2 amide NH protons were absent in the spectra for all diastereomeric product A (*anti*)/product B (*syn*) complexes. These results are consistent with technetium coordination at the expected Dap-Lys^2-Cys amino acids; the amide protons are lost during chelation.

In studies of [TcO] tripeptides,[22] characteristic NMR features have been identified that differentiate between the *syn* and *anti* diastereomers of technetium(V) oxo peptide complexes which have N_3S chelators of the type found in [TcO]depreotide. NMR results for the α and β protons of the cysteine bound to the metal are diagnostic for the two diastereomers. In particular, the Cys α protons of the *anti* diastereomers had a substantially higher chemical shift (5.57−5.75 ppm) than those of the *syn* diastereomers (5.20−5.26 ppm), and the Cys β protons of the *anti* diastereomer exhibited two resonances as compared to one resonance for the *syn* diastereomer. Table 1 summarizes the cysteine proton NMR chemical shifts for products A and B of both [^{99}TcO]depreotide and [^{99}TcO]P2540, along with typical ranges derived from the [TcO] tripeptide model compounds for comparison. The results clearly show that [^{99}TcO]depreotide and [^{99}TcO]P2540 have cysteine α and β proton NMR patterns that are consistent with the assignments made for the TcO tripeptides.

Table 1. ^{1}H NMR Chemical Shift (ppm) Results for Cysteine Protons in [^{99}TcO]Depreotide, [^{99}TcO]P2540, and [^{99}TcO]Tripeptides[a] Products A and B

product	Tc peptide	Cys Hα	Cys Hβ
A "*anti*"	TcO depreotide	5.58 (dd)	3.36, 3.62
	TcO P2540	5.73 (dd)	3.60, 3.80
	TcO tripeptides [range]	[5.57−5.75] (dd)	[3.48−3.57, 3.71−3.83]
B "*syn*"	TcO depreotide	5.20 (d)	3.80
	TcO P2540	5.29 (d)	3.80
	TcO tripeptides [range]	[5.20−5.26] (d or dd)	[3.80−3.87]

[a] Summary (range) of results taken from a separate publication[22] for $n = 6$, product A, TcO tripeptides and $n = 5$, product B, TcO tripeptides.

Table 2. Selected ^{1}H NMR Chemical Shift (ppm) Comparison of [^{99}TcO]Depreotide Products A and B to Free Depreotide[a,b]

proton	free peptide	product A (*anti*)	Δ_A	product B (*syn*)	Δ_B
Dap H_α	4.11	4.38	+0.27	3.73	**−0.38**
Lys^2 H_α	4.27	4.80	+0.53	4.49	+0.22
Lys^2 H_γ	1.30	0.65	−0.65	1.40	+0.10
Cys H_α	4.37	5.58	+1.21	5.20	+0.83
Cys H_β	2.80	3.36, 3.62	+0.56, +0.82	3.80	+1.00
Lys^4 H_α	4.09	4.10	+0.01	4.08	−0.01
Tyr H_α	4.56	4.54	−0.02	4.56	0.00
Trp H_α	4.19	4.15	−0.04	4.22	+0.03
Lys^{334} H_α	3.65	3.61	−0.04	3.63	−0.02
Val H_α	4.31	4.28	−0.03	4.21	−0.10
Hcy H_α	4.11	4.10	−0.01	4.25	+0.14
Hcy H_β	**−0.35, 1.16**	**−0.34, 1.18**	**+0.01, +0.02**	**−0.58, 1.23**	**−0.23, +0.07**
Phe H_α	5.08	5.05	−0.03	4.85	−0.23
Phe H_β	2.85, 3.19	2.81, 3.17	−0.04, −0.02	2.81, 2.92	−0.04, −0.27

[a] Δ = difference between Tc 99 complex and peptide values. [b] Bold values signify upfield shifts ascribed to anisotropic interactions.

Hence, we can conclude that for [TcO]depreotide and [TcO]-P2540, product A corresponds to the *anti* diastereomer, while product B corresponds to the *syn* diastereomer.

The absence of the NMR signals for Cys or Lys^2 amide NH protons indicates that the technetium coordination occurs at the expected Dap-Lys^2-Cys amino acids. Other NMR results provide further information concerning the influence of the metal binding site on the peptide. Table 2 summarizes chemical shift differences for selected protons in [^{99}TcO]depreotide products A and B relative to the free peptide. For protons removed from the technetium center (e.g., Lys^4 H_α, Tyr H_α, Trp H_α, Lys^{334} H_α, Val H_α, Hcy H_α), there is, in general, little change between the chemical shifts of the technetium complexes versus that of the free peptide. For protons in the vicinity of the technetium center (on the ligand backbone of the chelating amino acids Dap, Lys^2, and Cys), downfield shifts in the resonances relative to that in the free depreotide peptide are observed. Downfield shifts are expected when a ligand binds to a metal,[20,30] and therefore, these results further confirm that the site of Tc complexation is at the Dap-Lys^2-Cys sequence.

For protons located in the vicinity of the metal complexing site, there were two instances listed in Table 2 for [^{99}TcO]-depreotide (and in Table S4 for ^{99}Tc P2450) in which the peptide protons exhibit NMR signal shifts *upfield* upon complexation of the ligand with technetium. The Dap H_α resonance of *syn* [^{99}TcO]depreotide and the Lys^2 H_γ resonance of *anti* [^{99}TcO]-depreotide shift upfield by 0.38 and 0.65 ppm, respectively. We ascribe these upfield shifts to anisotropic interactions within the metal center and not to interaction of these chelator protons with pharmacophore aromatic residues for the following reasons: (a) The Dap α-proton and the Lys^2 γ-protons lie on opposite sides of the chelator plane; therefore, if the Dap alpha proton in the *syn* diastereomer was positioned to interact with a pharmacophore aromatic group, the Lys^2 side chain would be aimed out and away from the pharmacophore, which is at odds with ROESY results, indicating a Lys^2−Phe interaction (*vide infra*); (b) We also observe the same upfield shifts of the analogous protons (i.e., of the *syn* diastereomer Dap H_α and the *anti* diastereomer Lys^2 H_γ protons) in NMR results of the model peptide complex [^{99}TcO]P2540 and in model [^{99}TcO]-tripeptides bearing lysine in the second amino acid,[22] all of which contain no pharmacophore.

Further NMR analyses provide some insight into the conformation of the *syn* diastereomer of [^{99}TcO]depreotide. ^{1}H NMR ROESY results for *syn* [^{99}TcO]depreotide (product B) show ROE interactions between the pharmacophore ring phenylalanine and the lysine (Lys^2) of the technetium chelate (Phe Ar-3,4 ↔ Lys^2 H_γ, Phe Ar-3,4 ↔ Lys^2 H_ϵ, and Phe Ar-2 ↔ Lys^2 H_β). In addition, some protons on the pharmacophore of *syn* [^{99}TcO]depreotide exhibit upfield shifts in chemical shift values relative to the same protons on the free peptide (Table 2): the N-MePhe α-proton (shifted by 0.23 ppm), one of the Phe β-protons (shifted by 0.27 ppm), and one of the Hcy β-protons (shifted by 0.23 ppm). These upfield shifts at the pharmacophore protons could be explained by anisotropic interactions with the metal chelator. Hence, a picture emerges for *syn* [^{99}TcO]depreotide of a conformationally constrained, folded peptide in which the chelator is bent around toward the pharmacophore. Similar interactions (as indicated by ROESY or chemical shift changes) between protons on the cyclic pharmacophore and protons near the coordinated technetium were not observed for the *anti* isomer.

The constrained nature of the *syn* diastereomer may explain its increased receptor binding affinity relative to the anti diastereomer (*vide infra*). With a constrained, folded structure, the technetium chelator portion of the molecule is locked into a postion adjacent to the N-Me Phe-Hcy amino acids and is unable to interfere with the pharmacophore−receptor binding, which occurs at the opposite side of the pharmacophore peptide, at the Tyr-D-Trp-Lys^{334}-Val sequence. The constrained conformation of the *syn* species may also contribute to its stability relative to the *anti* product, resulting in the observation of ∼90/10 *syn*/*anti* equilibrium ratios obtained in the synthesis of both the ^{99}Tc and the ^{99m}TcO depreotide species.

No significant shifts or ROEs were noted in the proton resonances of the Tyr-D-Trp-Lys^{334}-Val region for the *syn* or *anti* diastereomers, indicating that the bioactive portion of the depreotide peptide undergoes no conformational changes as a result of complexation with Tc.

Circular Dichroism Spectroscopy of [^{99}TcO]Depreotide Isomeric Products A and B. Circular dichroism (CD) spectroscopy can also discern between *syn* and *anti* diastereomers of Tc peptides. The validation of the method, achieved using technetium tripeptide model complexes, is described in detail in a separate publication.[22] Figure 3 shows the CD data in methanol for both products A and B of [^{99}TcO]depreotide and [^{99}TcO]P2450. The visible region (where the peptide P2540 shows no features, Figure S7) is the most important region for characterization of the ^{99}Tc species because the visible region reports on the coordination about the ^{99}Tc center. The Tc tripeptide studies revealed that *anti* diastereomers possess a CD spectra containing a positive Cotton effect band occurring

Table 3. Biological Comparison Results for *Syn* [TcO]Depreotide, *Anti* [TcO]Depreotide, an *Anti*/*Syn* Mixture (7/93, from an Authentic [99mTcO]Depreotide Clinical Preparation), and Depreotide in AR42J Tumors[a]

	anti	*syn*	mixture	depreotide
		In Vitro Binding Affinity (Tc 99)		
IC_{50} (nM)	0.89 ± 0.15[b]	0.15 ± 0.04[b]	n.d.[c]	7.4 ± 2.6
		In Vivo Biodistribution (Tc 99m)		
tumor uptake (% ID/g)	3.38 ± 0.25[b]	6.58 ± 0.36[b]	5.34 ± 0.34[b]	n.d.[c]
blocked: + octreotide	1.58 ± 0.29[b]	2.12 ± 0.59[b]	1.04 ± 0.07[b]	n.d.[c]
tumor/blood	7.0	18.8	10.1	n.d.[c]
tumor/muscle	10.2	16.0	21.4	n.d.[c]

[a] Biodistribution: $n = 6$ Mice. [b] Results significantly different by student's t-test analysis. [c] n.d. = not determined.

between 300 and 400 nm and a broad asymmetrical band with a negative Cotton effect between 400−600 nm. Conversely, the *syn* diastereomers exhibit a broad negative Cotton effect band between 300 and 425 nm and a weak positive Cotton effect band centered at 460 nm. The CD results in Figure 3 and Figure S7 clearly confirm that products A and B of [99TcO]depreotide and [99TcO]P2540 correspond to the *anti* and *syn* diastereomers, respectively.

Biology. The binding affinities of *syn*/*anti* [99TcO]depreotide to SSTRs were evaluated using isolated AR42J rat pancreatic tumor membranes. AR42J tumors are known to express SSTRs in high density.[31] Tumor membranes were isolated from tumors grown subcutaneously in nude mice. The individual *syn*/*anti* isomers of the 99mTc tracer complex [99mTcO]depreotide were isolated for receptor binding and tumor biodistribution studies using preparative reversed phase HPLC. Isolated fractions had ≥80% radiochemical purity with ≥90% isomeric purity. Binding affinities of the 99Tc *syn* or *anti* isomeric species were determined by inhibition of specific binding of the isolated 99mTc tracer complexes [99mTcO]depreotide (*syn* or *anti*) with varying concentrations of each 99Tc complex. Tumor uptake biodistribution studies of *syn* and *anti* [99mTcO]depreotide were also conducted on nude mice with AR42J tumor xenografts. For comparison, an authentic clinical preparation of [99mTcO]-depreotide comprised of a mixture of the *syn* and *anti* species was also evaluated in vivo. Binding affinity and biodistribution results are given in Table 3.

Comparison of the binding affinities for the *syn* and *anti* isomers to that of uncomplexed depreotide shows significant increases in receptor affinity upon complexation of the peptide to the metal. These results are in keeping with previous reports for SSTR binding peptides and their rhenium complexes (for example, IC_{50} of [ReO]depreotide = 0.9 nM).[8] Furthermore, the results show that the *syn* isomer (product B in the 99Tc depreotide preparation) has a higher binding affinity than the *anti* isomer (IC_{50} = 0.15 nM vs 0.89 nM). Both binding affinity values are in the range of that for native somatostatin (IC_{50} = 0.1−1.3 nM for SSTR2).[32]

The improvements in receptor affinity may result from increasing conformational restriction of the peptide molecule. Formation of the technetium complex would restrict the movement of the peptide in the chelator portion of depreotide. Such constraints might limit interference of the pharmacophore−receptor interaction by the ancillary chelator peptide sequence, leading to a higher binding affinity value for the metal complex relative to the uncomplexed peptide. In the case of the *syn* isomer, the still higher observed binding affinity may be a result of further restriction introduced by folding of the molecule (vide supra) to move the chelator portion of depreotide toward the noncritical N-Me phenylalanine portion of the pharmacophore and homo cysteine linker, leaving the critical receptor-binding Tyr-D-Trp-Lys[334]-Val sequence of the pharmcophore[33] unperturbed.

There have been other reports of changes in receptor binding affinity in peptides and proteins upon metal complexation.[34,35] In a study of α-melanotropin peptide-based technetium/rhenium radiopharmaceuticals targeting melanoma,[35] the metal complex displayed a 2-fold increase in receptor binding affinity over the free peptide. This peptide was designed such that the complexed metal was incorporated into the molecule while maintaining a critical cyclic structural feature and without straining the pharmacophore sequence.

Results for DTPA and DOTA receptor-targeting peptides appear to be consistent with the hypothesis that conformational restriction introduced during metal complexation can lead to increases in receptor binding affinity. In general, DTPA octreotide peptides display improved binding affinities toward SSTR when complexed to indium.[36−38] In contrast, DOTA peptides appear to be less influenced by metal complexation. DOTA octreotide analog peptides when complexed to yttrium[39] or copper[40] exhibit marginally the same binding afiinities, and a DOTA bombesin analog peptide had a marginally improved binding affinity when complexed to lutetium.[41] When the polydentate ligand DTPA complexes to the metal, a significant amount of conformational freedom is lost and, like depreotide, the amount of interference with the pharmacophore−receptor interaction by the chelator is expected to decrease, leading to improved binding affinity. For the macrocyclic DOTA ligand, however, there is much less conformational restriction upon metal complexation because the macrocyclic ring is already constrained and, therefore, the receptor affinity is expected to be less affected. The differences noted between the metal complexes and the free ligands of the DTPA and DOTA octreotide derivatives might be related to other factors besides conformational restriction. For example, complexation of the metal by a DO3A (DOTA) chelator can lower the binding affinity of the ligand by restricting the proper orientation of D-Phe,[1,42] which is known to play a role in binding to SSTR. This deleterious restriction of the D-Phe[1] residue is expected for Y-DOTA complexes because they possess an eight-coordinate structure involving the carbonyl oxygen adjacent to D-Phe,[1] but it is not expected for In−DTPA complexes, where the metal is seven-coordinate and the analogous adjacent carbonyl oxygen is not involved in coordination.[43] Hence, Y-DOTA complexes might show less improvement in binding affinity upon metal complexation relative to In−DTPA complexes because they are subject to an additional negative effect imposed by the distortion of the D-Phe[1] residue.

The improved receptor affinity of *syn* relative to *anti* [99TcO]-depreotide is also reflected in the in vivo tumor biodistribution results, where the tumor uptake of the *syn* isomer is approximately twice that of the *anti* isomer (6.58% vs 3.38%). Excess octreotide (a known somatostain receptor binding peptide) was able to significantly block tumor uptake of both the *syn* and *anti* [99TcO]depreotide isomers (data not shown), indicating that tumor uptake of [99mTcO]depreotide is specifi-

cally mediated by upregulated SSTRs. Despite the differences in biological efficacy between the two isomers, both are expected to perform well as SSTR-positive tumor imaging agents, as evidenced by the high tumor-to-background (tumor/blood > 7, tumor/muscle > 10) ratios for both species.

Other literature reports of comparisons of the biological properties of *syn* and *anti* isomers of technetium(V) radiodiagnostic agents pertain to brain perfusion agents and renal functional imaging agents.[44] This paper constitutes the first report of observed differences in biological behavior between *syn* and *anti* isomers of a tumor-targeting agent. Moreover, it is the first example of such a comparison for a receptor-mediated radiopharmaceutical.

Conclusion

The radiolabeled product prepared from the "Kit for the Preparation of Technetium Tc 99m Depreotide Injection" has been confirmed to be a pair of diastereomeric *syn*/*anti* forms of the technetium(V) oxo complex [^{99m}TcO]depreotide, with the *anti* diastereomer eluting first on RP HPLC and the *syn* diastereomer (product B) eluting second. The NeoTect preparation contains *anti* [^{99m}TcO]depreotide and *syn* [^{99m}TcO]depreotide in an *anti*/*syn* ratio = ~10/90. NMR data provided insights into the conformation of the diastereomers. ROESY and chemical shift data indicate that the *syn* species has a folded conformation with the metal chelator positioned adjacent to the N-Me Phe-Hcy sequence of the pharmacophore.

The *syn* diastereomer had a higher SSTR affintiy in in vitro binding assays than the *anti* diastereomer and higher tumor uptake in tumor xenograft nude mouse biodistribution studies. Both diastereomeric technetium complexes had higher receptor binding affinities than the free peptide. The differences in receptor binding and tumor uptake may reflect improvements in binding of the molecule to SSTR resulting from conformational restrictions imposed by metal chelation and/or by selective folding of the *syn* diastereomer molecule. The receptor-binding portion of depreotide is not involved in the conformational changes.

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Supporting Information Available: Figures S1−S7 and Tables S1−S4 (15 pages); spectroscopy data of the compounds of interest. This material is available free of charge via the Internet at http://pubs.acs.org.

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