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***In Vitro* Structure-Activity Relationship of Re-cyclized Octreotide Analogues**

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Abstract

Introduction—Development of radiolabeled octreotide analogues is of interest for targeting somatostatin receptor-positive tumors for diagnostic and therapeutic purposes. We are investigating a direct labeling approach for incorporation of a Re ion into octreotide analogues, where the peptide sequences are cyclized *via* coordination to Re rather than through a disulfide bridge.

Methods—Various octreotide analogue sequences and coordination systems (e.g., S₂N₂ and S₃N) were synthesized and cyclized with non-radioactive Re. *In vitro* competitive binding assays with ¹¹¹In-DOTA-Tyr³-octreotide in AR42J rat pancreatic tumor cells yielded IC₅₀ values as a measure of somatostatin receptor affinity of the Re-cyclized analogues. Three-dimensional structures of Re-cyclized Tyr³-octreotate and its disulfide-bridged analogue were calculated from two-dimensional NMR experiments to visualize the effect of metal cyclization on the analogue's pharmacophore.

Results—Only two of the eleven Re-cyclized analogues investigated showed moderate *in vitro* binding affinity toward somatostatin subtype 2 receptors. Three-dimensional molecular structures of Re- and disulfide-cyclized Tyr³-octreotate were calculated, and both of their pharmacophore turns appear to be very similar with minor differences due to metal coordination to the amide nitrogen of one of the pharmacophore amino acids.

Conclusions—Various Re-cyclized analogues were developed and analogue **4** had moderate affinity toward somatostatin subtype 2 receptors. *In vitro* stable studies that are in progress showed

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stable radiometal-cyclization of octreotide analogues *via* NS_3 and N_2S_2 coordination forming 5- and 6- membered chelate rings. *In vivo* biodistribution studies are underway of $^{99\text{m}}\text{Tc}$ - cyclized analogue 4.

Keywords

Octreotide; Tyr³-octreotate; metal cyclization; Re; 2-D NMR

1. Introduction

The direction of radiopharmaceutical research has shifted toward developing site-specific targeting agents. Somatostatin receptors (SSTR) have gained much attention in the past 30 years due to their expression in high densities on neuroendocrine tumors. The somatostatin receptors belong to a family of seven transmembrane domain G protein-coupled receptors and are expressed on neuroendocrine tumors such as pituitary adenomas, pancreatic endocrine tumors, carcinoids, paragangliomas, pheochromocytomas, small cell lung cancers, medullary thyroid carcinomas, breast cancers, and malignant lymphomas [1,2].

Today, there are five known somatostatin receptor subtypes that have been cloned [2]. The majority of somatostatin receptor-positive tumors express multiple receptor subtypes, with a variation in their expression levels on different tumor types. It has been reported that the somatostatin subtype 2 receptor (SSTR2) is more abundant in tumors than the other subtypes and is expressed in more than 80% of endocrine pancreatic and endocrine digestive tract tumors [2]. Octreotide [(Sandostatin), DPhe-Cys-Phe-DTrp-Lys-Thr-Cys-Thr(ol)] was the first commercially available somatostatin analogue that possessed similar pharmacologic properties to the native somatostatin peptide [3–5].

Most studies on radiolabeling octreotide analogues with radiometals have used a bifunctional chelating approach, in which a radiometal is coordinated to a chelator that is covalently linked to the octreotide peptide. Perhaps the most well known of these is ^{111}In -DTPA-octreotide, marketed as OctreoScan[®] by Tyco Healthcare (Mallinckrodt, St. Louis, MO), which was also the first FDA-approved somatostatin receptor imaging agent [6]. In-111-DTPA-octreotide is now recognized to be “an important, if not the first imaging technique, chosen by physicians for localization and staging of neuroendocrine tumors through SPECT” [7]. It was reported that $^{99\text{m}}\text{Tc}$ -EDDA/HYNIC-Tyr³-octreotide had higher sensitivity as a clinical imaging agent when compared to ^{111}In -DTPA-octreotide [8,9]. However, non-specific uptake in the bowel and inflammatory lesions were observed with $^{99\text{m}}\text{Tc}$ -EDDA/HYNIC-Tyr³-octreotide [9], leading to the need to perform additional scanning to avoid false-positive findings. This procedure may produce patient compliance complications.

An alternative approach to the design of radiolabeled tumor imaging and therapeutic agents involves incorporating the radiometal directly into the molecular structure. A limited amount of work has been reported on Re- and $^{99\text{m}}\text{Tc}$ -cyclized somatostatin analogues developed for high affinity receptor binding and efficient tumor uptake. Our interest in investigating a direct labeling approach on octreotide analogues derives from previous work reported on the alpha-melanocyte-stimulating hormone (alpha-MSH) [Ac-Ser-Tyr-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH₂] [10]. Both labeling approaches, bifunctional chelating and integrated, were used to radiolabel alpha-MSH [10,11]. Metal-cyclized alpha-CCMSH, an alpha-MSH analogue containing three Cys residues, greatly improved the chemical stability of radiometal-peptide coordination, compared to a metal-cyclized two Cys-containing analogue. [11]. In addition to the stable metal coordination, higher receptor binding affinity and greater tumor uptake and retention were observed with the metal-cyclized/integrated alpha-CCMSH, as compared with the bifunctional chelating approach shown previously by our group [11–13].

We previously reported a series of Re-cyclized peptides, based on octreotide and Tyr³-octreotate [DPhe-Cys-Tyr-DTrp-Lys-Thr-Cys-ThrOH] sequences [14]. Re-cyclized Tyr³-octreotate (Re-TATE) had the lowest IC₅₀ value of the investigated peptides at 29 nM [14]. From 2-D NMR analysis of TATE and Re-TATE, the Re was found to be coordinated to two Cys sulfhydryls, the *N*-terminal nitrogen, and the amide nitrogen from Tyr³ (an amino acid that is part of the receptor-binding pharmacophore), as illustrated in Figure 1 [14]. The metal binding to one of the pharmacophore residues likely altered the analogue's receptor binding structure and reduced the binding affinity from 2 nM to 29 nM. The *N*-terminal amine coordination of the metal resulted in an unfavorable eight membered chelate ring, which may explain the observed instability of the ^{99m}Tc-cyclized Tyr³-octreotate analogue during the in vitro stability studies and in vivo animal imaging due to metal center oxidation and decomposition as has been previously published [15].

In this paper, the results of our structure-activity studies on a series of Re-cyclized octreotide analogues for targeting somatostatin receptors will be discussed. A series of modified peptide sequences were investigated in an attempt to improve both the receptor affinity and radiometal coordination stability of metal-cyclized octreotide analogues. The sequences contained such modifications as Cys replacement of DPhe to give three Cys peptides, sequence elongation by insertion of 1 to 2 amino acids, and use of synthetic bidentate Cys and DCys residues. The sequence changes affected the mode of metal coordination as well as the receptor binding affinity of the resultant metal-cyclized peptides. In addition, we report the results of our three dimensional (3-D) molecular structure calculations of previously synthesized disulfide- and Re-cyclized TATE [14], to visualize the effect of metal-cyclization on the receptor-binding pharmacophore.

2. Materials and methods

2.1.1 Materials

Reagents and solvents were purchased from VWR Scientific Products (St. Louis, MO), Novabiochem (San Diego, CA), Fluka (Milwaukee, WI), Fisher Scientific (Pittsburgh, PA), Sigma-Aldrich (St. Louis, MO), ACROS-Organics (Geel, Belgium), CEM (Matthews, NC), and BACHEM (Torrance, CA). All reagents and solvents were HPLC grade, peptide synthesis grade, or of the highest purity obtainable and were used without further purification.

2.1.2 Methods

RP-HPLC analysis and semi-preparative purification of macroscopic products were carried out on a Beckmann Coulter System Gold HPLC equipped with a 168 diode array detector, a 507e autoinjector, and the 32 KARAT software package (Beckmann Coulter, Fullerton, CA). Analytical RP-HPLC was performed on a Keystone Scientific, Inc. (Bellefonte, PA) C₁₈ Kromasil column (100 Å, 0.46 × 15 cm, 5 μm) with linear gradients of solvent B in solvent A (A: 0.1% TFA in water; B: 0.1% TFA in acetonitrile), a 1 mL/min flow rate, and UV detection at 214 and 280 nm. Semi-preparative RP-HPLC was performed on a Waters (Milford, MA) Prep Nova-Pak HR C₁₈ column (60 Å, 1.9 × 30 cm, 6 μm), also using linear gradients of solvent B in solvent A, but with flow rates up to 10 mL/min and UV detection at 225 and 280 nm. LC-ESI-MS analyses were carried out on a Thermo Finnigan LC system consisting of a P4000 quaternary LC pump and SCM1000 vacuum degasser, an AS3000 autosampler, and a UV6000LP diode-array detector connected to a Thermo Finnigan TSQ7000 triple-quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA). A Waters Nova-Pak C₁₈ column (60 Å, 0.39 × 30 cm, 4 μm) was used with a 1 mL/min flow rate and linear gradients of solvent B in solvent A. Radiotracer reactions were monitored and purified on a Waters 626 chromatograph RP-HPLC equipped with a Canberra NaI(Tl) well detector (Meriden, CT) for radioactivity detection and Waters 2487 Dual λ Absorbance Detector for UV detection at 214 and 280 nm.

Radio-RP-HPLC was performed on a Grace Vydac (Hesperia, CA) Protein & Peptide C₁₈ column (300 Å, 0.46 × 25 cm, 5 µm) attached to a Grace Vydac High Performance Guard Column. Linear gradients of solvent B in solvent A with a 1 mL/min flow rate were used.

2.2 Re-cyclization and Characterization

2.2.1 Synthesis of Reduced Linear Peptides—The linear reduced peptides (Table 1) were synthesized with a microwave-enhanced multiple peptide synthesizer (Liberty Automatic Microwave Peptide Synthesizer, Mathews, NC) using standard solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. Fmoc-Thr(tBu) Wang resin, 100–200 mesh, was used for all octreotide-derived peptides. The protected amino acids, as well as solutions for coupling and deprotecting reactions, were separately dissolved and arranged in specific vessels of the instrument. The protection groups chosen for the amino acid side chains were: tBu (Thr, Tyr, DOTA), Trt (Cys), and Boc (Lys, dTrp). The Fmoc protecting groups were removed in each cycle by treatment with *N*-hydroxybenzotriazole (HOBt) and piperidine. Recoupling was automatically performed for each amino acid. The *in situ* activation of Fmoc-amino acids was carried out using *O*-benzotriazole-*N,N,N',N'*-tetramethyl uronium hexafluorophosphate (HBTU), *N*-methylpyrrolidinone (NMP), and *N,N*-diisopropylethylamine (DIEA). The peptides were acetylated at the *N*-terminus using a capping mixture of 0.5 M acetic anhydride, 0.125 M DIEA, and 0.015 M HOBt in DMF. The peptidyl-resins were cleaved and deprotected in a single reaction with the following mixture: trifluoroacetic acid (TFA) (92.5%), water (2.5%), 3,6-dioxa-1,8-octanedithiol (DODT) (2.5%), and triisopropylsilane (TIS) (2.5%). Precipitation and multiple washings with diethyl ether gave the final crude linear peptides, which were then characterized by HPLC and LC-ESI-MS.

2.2.2 Re-cyclization and Purification—Re-cyclization of analogues **1** through **8** was accomplished *via* transchelation reactions of Re(V) from [ReOCl₃(OPPh₃)(SMe₂)] [16] to the reduced linear peptides using a previously reported method [14]. In brief, the reduced linear analogue (4.76–4.90 mmol) was dissolved in 1 mL of 62% aqueous methanol and the pH was adjusted to 8.1–8.3 using 0.1 M NaOH. Excess [ReOCl₃(OPPh₃)(SMe₂)] (7.14–7.35 mmol) was added, and the reaction was heated for 30 min at 65 °C in a thermomixer. The reaction changed gradually from a mint green suspension to a peach-brown solution with a dark grey precipitate. The reaction mixture was centrifuged and filtered. LC-ESI-MS was employed to confirm the formation of the desired complex, which was then isolated by semi-preparative RP-HPLC. The purity, as determined *via* LC-ESI-MS, was >95% for all Re-cyclized analogues. The peptide complexes were then lyophilized and stored in the freezer until needed for further studies.

Re-cyclization of analogues **9** through **11** was carried out *via* transchelation reactions of Re (V) from tetrabutylammonium tetrachlorooxorhenium(V) (TBA)[ReOCl₄] [17] to reduced linear peptides using modified literature procedures [18,19]. Analogues **6–8** were synthesized by both methods described. A Schlenk line setup was employed due to the moisture-sensitive nature of the Re starting material. The reduced linear peptide (20.0–21.7 mmol) and excess [TBA][ReOCl₄] (60.0–65.1 mmol) were added to an argon-filled round bottom flask and were dissolved in 1 mL of anhydrous DMF. The color of the reaction ranged between yellow and green, depending on the peptide used. Each reaction was stirred at room temperature under positive pressure of argon overnight. The solvent was removed *via* a high pressure vacuum pump, and the resulting green colored glaze product was dissolved in a 1:5 acetonitrile:water solution. The resulting peach-brown solution was centrifuged and filtered to remove the presence of a dark grey precipitate. LC-ESI-MS was employed to confirm the formation of the desired complex, which was then isolated by semi-preparative RP-HPLC. The purity, as determined by LC-ESI-MS, was >95% for all Recyclized analogues. The peptide complexes were then lyophilized and stored in the freezer until needed for further studies.

2.2.3 2-D NMR Experiments of Re-cyclized Analogue 4—NMR spectra of Re-cyclized analogue **4** were collected on a Varian Unity Inova 600 MHz spectrometer equipped with a 5 mm [^1H , ^{15}N , ^{13}C] triple-resonance cold probe. NMR experiments, 2-D ^1H - ^1H total correlated spectroscopy (TOCSY) (80 ms mixing time), ^1H - ^1H nuclear Overhauser effect spectroscopy (NOESY) (200 ms mixing time), and ^1H - ^{13}C heteronuclear single quantum coherence (HSQC), were performed for a solution of 2.26 mM of Re-cyclized analogue **4** in 400 μL of 9:1 $\text{H}_2\text{O}/\text{D}_2\text{O}$. The Re-cyclized complex solution was prepared in a SHIGEMI NMR tube (Shigemi Co., Japan) and the experiments were carried out at 25 °C. NMR data were processed with NMRPipe [20] and analyzed in SPARKY software (Goddard, T.D. and Kneller, D.G., SPARKY, University of California, San Francisco). Indirect dimensions were normally extended by linear prediction and zero-filled prior to Fourier transformation, and only spectral regions containing signals were retained. The ^1H and ^{13}C chemical shifts were referenced to 2,2-dimethylsilapentane-5-sulfonic acid (DSS) as an external standard [21].

2.3 In Vitro Receptor Binding Assays (IC₅₀ Studies)

2.3.1 Preparation of Disulfide-cyclized ^{111}In -DOTA-TIDE—The disulfide-cyclized DOTA-TIDE was prepared as previously described [14]. Briefly, 2.8 mL of a 1:1 water/ acetonitrile (v/v) solution, 8.5 mL of 0.2 M ammonium acetate, and 1.7 mL of DMSO were added to 13 mg of linear DOTA-TIDE. The 1 mg/mL mixture was shaken overnight, resulting in near quantitative yield of the disulfide-cyclized DOTA-TIDE. The product was isolated by semi-preparative RP-HPLC, characterized by LC-ESI-MS, and subsequently lyophilized (purity of >98%). The radiolabeling of DOTA-TIDE was carried out following a modified literature procedure [22], using $^{111}\text{InCl}_3$ obtained from Mallinckrodt Medical (St. Louis, MO). In short, to 100 μL of a 30 mM sodium acetate/25 mM sodium ascorbate solution (pH 5.0) were added 50 μL of $^{111}\text{InCl}_3$ and 1 μg of disulfide-cyclized DOTA-TIDE, and the mixture was heated for 30 min at 99 °C. The ^{111}In -DOTA-TIDE was purified by RP-HPLC using a 1 mL/min flow with a 0–50% linear gradient of solvent B in solvent A over 30 min.

2.3.2 Cell Culture—AR42J rat pancreatic carcinoma cells, known to express SSTR2, were obtained from the American Type Culture Collection (Manassas, VA) and initially maintained by the Cell and Immunobiology Core Facility at the University of Missouri-Columbia. Monolayer cell cultures were maintained by serial passage in exponential growth phase in RPMI 1640 medium (GIBCO-Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 48 $\mu\text{g}/\text{mL}$ gentamycin, in a humidified atmosphere of 5% CO_2/air at 37 °C. Cell viability was determined to be >98% by trypan blue exclusion and hemacytometry. Prior to cell experiments, the growth media was removed and the cells were gently washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline. Following removal of the phosphate-buffered saline, the cells were trypsinized with 5 mL of TrypLE Express (GIBCO-Invitrogen, Carlsbad, CA). Trypsin was quenched by the addition of complete RPMI 1640 Medium (GIBCO-Invitrogen, Carlsbad, CA), supplemented with 10–20% fetal bovine serum. The cells were collected by centrifugation, the supernatant was removed, and the cells were resuspended in complete medium and counted.

2.3.3 IC₅₀ Studies—The *in vitro* SSTR2 binding affinities (IC₅₀ values) of the Re-cyclized analogues were determined from competitive binding assays with ^{111}In -DOTA-TIDE in AR42J tumor cells using a modified literature procedure [23]. The trypsinized cells were suspended in fresh media (RPMI 1640) at a concentration of 1×10^7 cells/mL. A Re-cyclized analogue solution was added to wells containing 2×10^6 AR42J cells/well with a fixed amount of ^{111}In -DOTA-TIDE (0.2 $\mu\text{Ci}/\text{well}$). Triplicate wells were made for each Re-cyclized analogue concentration used, which ranged from 10^{-4} to 10^{-13} M. After 2-h incubation in a humidified atmosphere of 5% CO_2/air at 37 °C, the cell-bound radioactivity was separated by centrifugation (1 min at 10,000 rpm) and aspiration of the incubation media. The cell pellets

were subsequently cooled on an ice block and washed three times with 4 °C fresh media (RPMI 1640), to remove any unbound or loosely bound radioactivity on the cells. The amount of ^{111}In -DOTA-TIDE bound to the cells was determined by measuring the radioactivity of the cell pellets on a Wallac 1480 Wizard 322 automated counter (PerkinElmer Life Sciences, Gaithersburg, MD). The percent of ^{111}In -DOTA-TIDE bound to the cells versus the log of Re-cyclized analogue concentration was plotted using GraFit software (Version 4.0.10, Erithacus Software Ltd., Horley, Surrey, UK) to generate the IC_{50} sigmoidal curve. The IC_{50} values are the concentrations of the Re-cyclized analogues that caused half-maximal inhibition of ^{111}In -DOTA-TIDE binding to the receptors

2.4 Three-dimensional molecular structure calculations

2.4.1 NOE Assignments and Bond Distance Calibrations—Proton-proton distance constraints of the highest binding SSTR2 affinity octreotide analogues previously investigated in our laboratories, disulfide- and Re-cyclized TATE [14], were derived from the intensities of the cross peaks on 2D ^1H - ^1H NOESY. Assignments of the nuclear Overhauser effect (NOE) cross peaks on NOESY spectra were made based on chemical shift assignments determined from ^1H - ^{13}C HSQC and ^1H - ^1H TOCSY and NOESY spectra. The NOE cross peak intensities were calibrated into distances based on the known distance of 2.8 Å for the Trp beta (β) proton to the delta (δ) proton, which is considered a medium range distance [24]. The height intensities of the NOEs are proportional to the inverse sixth power of the ^1H - ^1H distance [24]. The NOEs were classified as weak (W), medium (M), or strong (S), with corresponding upper constraint limits of 6.0, 3.5, and 2.7 Å, respectively.

2.4.2 NMR Restraints and Structure Calculations—The structure calculations were performed using X-PLOR-NIH [25], a computational program that requires information about the molecular structure and experimental NOE-derived distances. The molecular information of each of the amino acids and information about the peptide analogues must be generated using the all-hydrogen force fields that are identified in the “topallhdg.pro” and “parallhdg.pro” protocols for proteins. In the case of the disulfide-cyclized analogues, all known disulfide-bridge distance constraints were included in the simulated annealing protocol.

All observed NOE interactions were sorted according to the secondary structure conformation in which the NOE proton pair is located. The structures were calculated by using distance-restrained simulated annealing protocols and the X-PLOR-NIH program. Structure calculations of the three analogues were run using an experimentally restrained simulated annealing protocol including only the backbone NOEs of each conformation. This protocol generates molecular structures by heating the molecule to a set temperature followed by slow cooling of the molecule, with the number of structures minimized by eliminating those that violate the experimental NOE constraints. The procedure is repeated again and again to generate a large ensemble of conformations. The initial simulated annealing temperature was set at 1000 K with 6000 total steps at high temperature and 3000 total steps during the cooling process. The structures were evaluated at the final temperature of 300 K with 50 K for each step. The NOE distance restraints were represented by a soft-sum-well potential. Only structures that did not violate the distance restraint by >0.5 Å were selected by the simulated annealing protocol.

The generated structures of the disulfide-cyclized analogues from the simulated annealing protocol were refined *via* a refinement protocol set at an initial temperature of 3000 K with 4000 total steps at high temperature and 3000 total steps during the cooling process. The structures were again evaluated at a final temperature of 300 K with 50 K for each step. The NOE distance restraints were represented by a square-sum-well potential, and only structures that did not violate the distance restraint by >0.2 Å were selected by the refinement protocol.

The resulting structures for each of the analogues of interest were visualized *via* the Pymol computer program (DeLano Scientific LLC, San Carlos, CA).

3. Results and Discussion

3.1 Re-cyclization and Characterization

From the observed instability of ^{99m}Tc -cyclized TATE at the radiotracer level [15], further modifications of the peptide sequences were warranted, to both tighten the metal coordination and distance it from the pharmacophore. Direct metal cyclization of an alpha-MSH peptide containing two Cys residues resulted in an improved receptor binding affinity compared to the disulfide-cyclized counterpart [26]. In this study, the metal was reported to coordinate to the two thiolate sulfurs of Cys⁴ and Cys¹⁰, the amide nitrogen of Cys¹⁰, and the amide nitrogen of Trp⁹, with the Trp⁹ required for receptor binding. Giblin *et al.* suggested that the metal coordination contributed to the lower receptor binding affinity [26]. Therefore, a third Cys residue was added at the *N*-terminus, yielding a second generation metal-cyclized peptide (Re-alpha-CCMSH), with high receptor binding, tumor uptake, and tumor retention [26]. The metal coordinated to the three Cys thiolate sulfurs and a Cys amide, apparently distancing the metal coordination from the receptor binding portion of the peptide and restoring the receptor binding affinity.

We investigated metal-cyclization of analogues **1** through **11**, which each included an acetylated *N*-terminus, to disfavor the metal coordination to the *N*-terminal amine that was found for Re-cyclized TATE [14]. The DPhe in the first position was replaced by a third Cys in analogues **1** through **8** (Table 1), to draw the metal coordination sphere away from the receptor pharmacophore. Analogues **1** through **4** had either Phe or Tyr at the third position, with either an alcohol or carboxylic acid *C*-terminus. Analogue **5** differed from analogue **4** only in that analogue **5** had a DCys in the first position, to determine the importance of the stereochemistry of the first amino acid, since the position was originally occupied by DPhe. In analogue **6**, an amino acid was added between Cys² and Tyr³ as a spacer between the metal-coordinating Cys² and the pharmacophore. Two single amino acid spacers were added in both analogues **7** and **8**, between Cys² and Tyr³ and Thr⁶ and Cys⁷. The spacer amino acids added to analogues **6**, **7** and **8** were originally present in the natural occurring somatostatin-14 peptide. Analogues **7** and **8** differed only in the stereochemistry of the Cys in the first position. It was reported by Rivier and coworkers that DPhe¹, DTrp⁴, and Lys⁵ are the essential residues for octreotide analogues for binding to somatostatin subtype 2 receptors [27]. In analogues **1** through **8**, the DPhe in the first position was replaced by L/DCys. Therefore, to maintain the DPhe in the first position and explore the importance of this residue in receptor affinity, a third Cys was inserted between DPhe¹ and Cys² in analogue **9** and between Cys⁷ and Thr⁸ in analogue **10**. In order to distance the metal coordination site from the receptor pharmacophore without replacing DPhe¹ or elongating the sequence, the Cys in the second position was replaced with 3-aminopropylcysteine (Apc), to generate analogue **11** (Table 1). Apc is a commercially available bidentate Cys derivative that has a thioether and an amine for metal coordination, separated by a three carbon backbone.

Each of these modifications was made in an attempt to develop an octreotide analogue with *in vivo* stability without sacrificing receptor binding affinity, by tightening the metal coordination and distancing the metal core from the receptor binding amino acids. The reduced linear octreotide analogues **1** through **11** were synthesized *via* standard solid-phase Fmoc peptide chemistry. After cleaving the peptides from the resins and deprotecting the side chain protecting groups, the crude analogues were characterized *via* LC-ESI-MS. The calculated masses for the reduced linear analogues correlated with the observed masses from LC-ESI-MS, demonstrating the successful synthesis of each of the sequences (Table 2).

The reduced linear analogues **1** through **5** were successfully Re-cyclized *via* transchelation reactions with $[\text{ReOCl}_3(\text{OPPh}_3)(\text{SMe}_2)]$ in aqueous methanol solutions. Analogues **6–8** were Re-cyclized by transchelation reactions using both the $[\text{ReOCl}_3(\text{OPPh}_3)(\text{SMe}_2)]$ starting material as above, as well as $[\text{TBA}][\text{ReOCl}_4]$ in anhydrous DMF. The transchelation reaction was possible with both starting materials since Re was already in the +5 oxidation state. The same product was observed for both Re starting materials. Analogues **9** through **11** were Re-cyclized *via* transchelation reactions with $[\text{TBA}][\text{ReOCl}_4]$. Each of the Re-cyclized analogues was characterized using LC-ESI-MS, and the peaks with the expected masses had the predicted $^{185/187}\text{Re}$ isotopic pattern. Semi-preparative RP-HPLC was conducted using an optimized solvent multi-step gradient, determined from analytical RP-HPLC runs, that allowed for separating the desired peak from impurities. The collected fractions were characterized *via* LC-ESI-MS, and those with experimental molecular weights correlating with the calculated molecular weights were combined. The purity of Re-cyclized analogues was verified using LC-ESI-MS (Table 2), and the combined pure fractions (>95%) were lyophilized to dryness and stored at $-20\text{ }^\circ\text{C}$ until needed for *in vitro* studies.

In order to determine the metal coordination of the analogues containing three Cys residues (analogues **1** through **10**), the metal coordination of the Re-cyclized analogue **4** was determined by assigning the ^1H and ^{13}C chemical shifts from the ^1H - ^{13}C HSQC, ^1H - ^1H TOCSY, and ^1H - ^1H NOESY spectra (Table 3). The observed NMR chemical shifts of Recyclized analogue **4** were compared with those of the disulfide-cyclized acetylated-TATE (NTATE) previously reported by our group [14]. Downfield shifts, ranging between 0.46 and 0.74 ppm, of the βH s for Cys¹, Cys², and Cys⁷ were observed for Re-cyclized analogue **4** as compared to the βH chemical shifts of the disulfide-cyclized NTATE. In addition, the αH s of Cys¹ and Cys² were observed to have downfield chemical shifts of 0.32 and 0.76 ppm, respectively, with an upfield shift of 0.32 ppm found for the αH of Cys⁷ compared to the αH chemical shift of Cys⁷ residues in disulfide-cyclized NTATE. These chemical shift alterations of the αH s and βH s of Cys¹, Cys², Cys⁷ suggest that the thiolate sulfurs of these three Cys residues had coordinated to the Re metal center.

NMR cross peaks from backbone amide proton of Cys⁷ were absent from the TOCSY and NOESY spectra of the Re-cyclized analogue **4**. This result, coupled with the significant downfield shifts of the αH to 5.38 ppm and of the αC to 65.17 ppm observed for Cys² of Re-cyclized analogue **4** indicate that the metal was coordinated to the amide nitrogen of Cys². Therefore, the NMR data indicates that the metal center in Re-cyclized analogue **4** coordinated, as expected, to the three Cys thiolates and the amide nitrogen of Cys². As shown in Figure 2, this metal coordination mode results in the formation of a six- and five-membered bicyclic ring. A similar coordination mode was reported for the Re-cyclized alpha-CCMSH peptide [26].

From the analysis of the 2-D TOCSY NMR spectrum, it was observed that the cross peaks from the amide protons of Tyr³ and Lys⁵ were missing (Table 3). However, the observed chemical shifts of the αH and βH_2 of Tyr³ and Lys⁵ do not indicate that their backbone amides were involved in metal coordination; instead, the absence of the cross peaks are likely due to line-broadening caused by increased backbone mobility of the flexible loop in the 21-membered cyclic ring region of the peptide. Also, the presence of a second ^1H chemical shift set for the terminal amino acids (the *N*-terminal CH_3 of Cys¹ and the αH of Thr⁸) indicated that multiple conformations were present toward both termini, suggesting that the metal coordination restricted the peptide's overall flexibility in the region involved in metal coordination and only the termini had the ability to fluctuate resulting in the detection of multiple conformations.

3.2 In Vitro Receptor Binding Assay (IC₅₀ Studies)

The concentrations of Re-cyclized analogues **1** through **11** that resulted in half-maximal inhibition (IC₅₀ values) of the radiolabeled standard binding to SSTR2 were determined from *in vitro* competitive receptor binding studies performed with SSTR2-expressing AR42J rat pancreatic tumor cells. The program GraFit was used to plot the data and obtain the IC₅₀ curves and values (Table 2). The IC₅₀ values of the Re-cyclized analogues **2** and **4** were 4.6 and 3.4 μ M, respectively, while the remaining analogues had values that were >100 μ M. These values are significantly higher than the range of the first generation of Re-cyclized peptide sequences, meaning that the modifications made to generate the series of analogues reported herein actually reduced the binding affinity to the SSTR2. In such a small peptide, this relatively rigid structure, which involves four atoms of the peptide backbone, may put significant strain on the backbone near the site of metal coordination that in turn affects the backbone of the nearby pharmacophore residues.

Re-cyclized analogues **1** through **4** are each eight amino acids long and differ only in their amino acid in position three and their C-termini. Therefore, it is reasonable to predict that each of these analogues coordinated the Re metal center as determined for analogue **4**. The formation of the six-, five-membered bicyclic ring with the metal center, as shown in Figure 2 for Re-cyclized analogue **4**, may have caused a strain on the backbone of the peptides leading to a distortion of the receptor binding pharmacophore and the resulting loss of receptor affinity. However, the same metal coordination in the Re-cyclized alpha-CCMSH peptide did not seem to hinder its receptor affinity, yet this may be explained by the longer sequence (ten amino acids versus eight) of this peptide.

The importance of the stereochemistry of the first amino acid in the peptide sequence for receptor affinity was explored with Re-cyclized analogues **4** and **5**. The single difference between analogues **4** and **5** was the stereochemistry of Cys¹; analogue **4** has an LCys while analogue **5** has a DCys (Table 1). The lower receptor binding affinity (>100 μ M) of Recyclized analogue **5** compared to Re-cyclized analogue **4** (3.4 μ M) and the Cys¹ involvement in metal coordination, suggests a negative effect of the DCys on the receptor binding affinity. This may not have been the case if the Cys¹ was not involved in metal coordination, as a similar effect was not seen in the case of the metal-cyclized TATE, which contained a DPhe at the first position. Furthermore, we cannot conclude whether or not the stereochemistry of the first amino acid is of importance for receptor binding, since only one such pair of analogues was studied and their large IC₅₀ values make conclusive comparisons difficult, due to the inherent error in such high values. The addition of spacer amino acids into analogue **4** to generate the metal-cyclized analogues **6** through **8** was intended to alleviate the proposed strain caused by the six-, five-membered bicyclic rings in analogues **1** through **5**. However, the elongation of the peptide sequence did not result in improved binding affinity to the receptors as anticipated. Therefore, the low receptor binding affinity of metal-cyclized analogues **1** through **8** might be due to more than just backbone strain.

From the study of Re-cyclized analogues **9** and **10**, the inclusion of DPhe in the first position of Tyr³-octreotate analogues with three Cys residues also did not improve the affinity toward the somatostatin subtype 2 receptors. Therefore, the loss of receptor binding affinity of the analogues containing three Cys residues (**1** through **8**) is not solely due to the absence of DPhe at the first position. Finally, for Re-cyclized analogue **11**, the bulk of the three carbon backbone between thioether and amine of the bidentate Cys once coordinated to Re may have distorted the receptor binding site, lowering its ability to bind to the receptor.

3.3 Three-dimensional Molecular Structure Calculations for TATE and Re-TATE

The three dimensional molecular structures of the previously investigated disulfide- and Re-cyclized TATE [14] were calculated due to the interest of determining the effect of metal cyclization of the best octreotide analogue investigated by our laboratory to date. The NOE height intensities of the disulfide- and Re-cyclized TATE compounds [14] were converted into distances and classified as weak, medium, or strong with corresponding upper constraint limits of 6.0, 3.5, and 2.7 Å, respectively. The NOE patterns were analyzed to predetermine the secondary structures, based on Wuthrich's published table of secondary structures in proteins by NMR [28]. All observed NOE interactions were sorted according to the secondary structure element in which the NOE proton pair is located.

Comparing the backbone NOEs observed for disulfide-cyclized TATE to the Wuthrich table indicated that a mixture of both beta-sheet and distorted helical backbone conformations were present. Therefore, the backbone NOEs were sorted into two distinct tables, for the beta-sheet and distorted helical conformations. The terminal amino acids were observed to define the overall structural conformation. Residues 3 through 6 of the peptide sequence are involved in the beta turn, the receptor pharmacophore, so only the terminal amino acids were left for structural conformations.

The presence of strong sequential alpha-amide proton ($H_i^{\alpha}-H_j^N$) NOEs (where i and j indicate the first second residues, respectively, involved in a cross peak) and weak range sequential amide-amide proton ($H_i^N-H_j^N$) NOEs indicate the presence of a beta-sheet conformation.

Strong to medium range sequential alpha-amide proton ($H_i^{\alpha}-H_j^N$) NOEs of residue pairs 1 and 2, 2 and 3, 6 and 7, and 7 and 8 were observed. Also, medium to weak range sequential amide-amide proton ($H_i^N-H_j^N$) NOEs for the beta-sheet conformation of disulfide-cyclized TATE were observed. Table 4 lists the NOE patterns for disulfide-cyclized TATE, which indicate the existence of the beta-sheet conformation.

Conversely, the presence of weaker range sequential alpha-amide proton ($H_i^{\alpha}-H_j^N$) NOEs, medium range sequential amide-amide proton ($H_i^N-H_j^N$) NOEs, and weak intensity long range alpha-amide proton NOEs, such as ($H_i^{\alpha}-H_{i+3}^N$) and ($H_i^{\alpha}-H_{i+2}^N$), indicate the existence of a helical structure. Since the helical portion of the disulfide-cyclized TATE was observed only toward the C-terminus of the peptide, between residues 5 and 8, this structure is referred to as a distorted helical conformation. Table 4 presents the helical portion of the backbone NOEs of the disulfide-cyclized TATE.

A turn is composed of four amino acids, and residues 3 through 6 are involved in the disulfide-cyclized TATE turn. The observed pattern of the backbone-backbone NOEs of the pharmacophore turn belongs to a type I' turn; the prime is due to the D-amino acid, an unnatural amino acid, involved in the turn. The NOEs between residues 3 through 6 belonging to the type I' turn are listed in Table 5.

Disulfide-cyclized TATE has two conformations at equilibrium, as was evident from the types of NOEs observed, which is consistent with what has been previously reported on the extensively investigated disulfide-cyclized octreotide [29–35]. Only backbone-backbone NOEs were used as experimental constraints in the structural calculations reported herein. The side chain NOEs were not included in the calculations since the intensities of the cross peaks may be averaged from the two conformations at equilibrium, making it difficult to sort the side chain and long range NOEs. In addition to the overlapping of similar cross peaks between conformations, some cross peaks may belong to only one of the conformations. One of each

of the calculated beta-sheet and distorted helical conformations of disulfide-cyclized TATE are shown in Figure 3. Despite the exclusion of the side chain NOEs, the resulting calculated structures of the disulfide-cyclized TATE are similar to the beta-sheet and distorted helical structures published for octreotide [35].

From HSQC and TOCSY spectral analysis of Re-cyclized TATE, two distinct sets of peaks were observed, which indicate the presence of two different structures of the Recyclized TATE. These sets of peaks might indicate the presence of *anti* and *syn* conformations due to the presence of the oxo group on the Re metal. However, only one set of peaks had sufficient backbone-backbone NOEs in NOESY for use in the 3-D molecular structure calculations. The initial analysis of the NOEs suggested that the major conformation of the complete set of NOEs favored a beta-sheet conformation (Table 6). Since the Re coordinated to the thiolate sulfurs of residues 2 and 7, the amide nitrogen in residue 3, and the amine nitrogen at the *N*-terminus, it was expected and observed that some of backbone NOEs involved with those residues would be missing, due to the loss of several protons on coordination to the metal center. The sequential alpha-amide proton ($H_i^{\alpha}-H_j^N$) NOEs between residue pairs 1 and 2, 6 and 7, and 7 and 8 were also weaker than those observed for the disulfide-cyclized counterpart, which indicates an effect of metal-cyclization on the overall structure conformation. As for the turn, not all of the NOEs in the disulfide-cyclized TATE were observed with the Re-cyclized counterpart, as can be seen by comparing Tables 5 and 6. Again, the loss of NOEs associated with the amide nitrogen proton of residue 3, which is lost during metal coordination, was expected, as this residue is a part of the pharmacophore. Involvement of the turn in metal coordination could certainly lead to a difference in the turn type; so it was not surprising that the observed NOEs of the turn were not consistent with a particular type of turn. Therefore, only the beta-sheet and turn backbone NOEs of Re-cyclized TATE were used in the 3-D molecular structure calculations, which in turn meant using fewer constraints than for the disulfide-cyclized counterpart (Figure 4). Only the simulated annealing protocol was used for the initial structure calculations, since the presence of the metal in the protocol made the calculations challenging.

The TATE peptide sequence appears to have a greater preference for the beta-sheet conformation structure as the metal-cyclized TATE, compared to the beta-sheet and distorted helical conformations at equilibrium in the disulfide-cyclized TATE. The metal coordination might be constraining the flexibility of the molecular structure of the octreotide analogue since the metal coordinated to two of the backbone nitrogens. The loss of high receptor binding affinity of our investigated Re-cyclized analogues might be due to the possibility of conformation restriction similar to that observed for Re-cyclized TATE. Metal cyclization might have caused the peptides to lose one of their conformations, and this loss might have been the structural conformation preferred by the receptors.

4. Conclusions

A series of octreotide analogues were developed that differed in their sequences and subsequently their metal coordination modes, in an attempt to attain a metal-cyclized octreotide analogue with both high binding affinity toward somatostatin subtype 2 receptors and stable metal coordination at the radiotracer level. Conditions were developed for the reproducible synthesis and purification of the Re-cyclized octreotide analogues. However, the modifications attempted herein did not result in an analogue with improved somatostatin receptor subtype 2 binding affinity than the Re-cyclized TATE peptide from our first generation of metal-cyclized octreotide analogues. While the other four somatostatin receptor subtypes were not investigated in these studies, it may be worth pursuing in the future to determine if the modifications explored herein simply altered the somatostatin subtype preference. Though the subtype 2 receptor affinity of these analogues was not improved, the modified metal coordination modes should lead to greater stability of the ^{99m}Tc -cyclized complexes. Radiotracer ^{99m}Tc labeling,

in vitro stability and *in vivo* biodistribution studies with the ^{99m}Tc -cyclized analogues are underway and will be reported shortly.

Disulfide-cyclized TATE has two molecular structures, beta-sheet and distorted helical conformations, at equilibrium. However, the introduction of a Re metal into the disulfide bridge constrained the flexibility of the molecule causing it to favor the beta-sheet conformation over the distorted helical conformation. From the backbone NOEs, as well as the 3-D molecular structure determined, the metal cyclization did not drastically affect the pharmacophore turn, which explains the only slightly reduced receptor binding affinity observed for Re-cyclized TATE. More studies are needed to fully understand the effect of metal cyclization of octreotide analogues on their receptor binding affinities.

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Figure 1.
Metal coordination of Re-cyclized TATE [14].

Figure 2.
Metal coordination of Re-cyclized analogue 4.

Figure 3.
Example calculated structures of the beta-sheet (a) and distorted-helical (b) conformations of disulfide-cyclized TATE.

Figure 4. Example calculated structure of the beta sheet conformation of Re-cyclized TATE. The Re metal is circled.

Table 1

Sequences of synthesized octreotide analogues

Analogues	Sequences
DOTA-TIDE	DOTA-DPhe-Cys-Tyr-DTrp-Lys-Thr-Cys-Thr(ol)
TATE [14]	DPhe-Cys-Tyr-DTrp-Lys-Thr-Cys-Thr(OH)
1	Ac-Cys-Cys-Phe-DTrp-Lys-Thr-Cys-Thr(ol)
2	Ac-Cys-Cys-Tyr-DTrp-Lys-Thr-Cys-Thr(ol)
3	Ac-Cys-Cys-Phe-DTrp-Lys-Thr-Cys-Thr(OH)
4	Ac-Cys-Cys-Tyr-DTrp-Lys-Thr-Cys-Thr(OH)
5	Ac-DCys-Cys-Tyr-DTrp-Lys-Thr-Cys-Thr(OH)
6	Ac-Cys-Cys-Asn-Tyr-DTrp-Lys-Thr-Cys-Thr(OH)
7	Ac-Cys-Cys-Asn-Tyr-DTrp-Lys-Thr-Ser-Cys-Thr(OH)
8	Ac-DCys-Cys-Asn-Tyr-DTrp-Lys-Thr-Ser-Cys-Thr(OH)
9	Ac-DPhe-Cys-Cys-Tyr-DTrp-Lys-Thr-Cys-Thr(OH)
10	Ac-DPhe-Cys-Tyr-DTrp-Lys-Thr-Cys-Cys-Thr(OH)
11	Ac-DPhe-Apc-Tyr-DTrp-Lys-Thr-Cys-Thr(OH)

Table 2

Characterization of analogues: MW of calculated vs. experimental linear reduced and Re-cyclized analogues and their IC₅₀ values. The MW reported for the Re-cyclized analogues are based on ¹⁸⁷Re mass. IC₅₀ values were determined against an ¹¹¹In-Tyr³-octreotide standard in AR42J pancreatic rat cells (n = 3)

Analogue	Linear Reduced		Re-cyclized		IC ₅₀ of Re-cyclized (µM)
	Calc. MW (M+H) ⁺	Exp. MW (M+H) ⁺	Calc. MW (M+H) ⁺	Exp. MW (M+H) ⁺	
TATE					29 nM [14]
1	1019.2	1019.7	1219.5	1219.5	>100
2	1035.2	1035.7	1235.5	1235.6	4.6 ± 0.7
3	1033.2	1033.4	1231.4	1231.6	>100
4	1049.2	1049.6	1249.3	1249.4	3.4 ± 0.9
5	1049.2	1049.4	1249.3	1249.6	>100
6	1163.3	1163.5	1361.5	1361.4	>100
7	1250.4	1250.8	1448.6	1448.3	>100
8	1250.4	1250.7	1448.6	1448.2	>100
9	1196.5	1196.2	1396.4	1396.1	>100
10	1196.5	1196.3	1396.4	1396.2	>100
11	1019.2	1019.7	1350.4	1350.8	>100

Table 3¹H and ¹³C NMR chemical shift assignments of Re-cyclized analogue **4**

Residue	NH	α CH	β CH	Others
<i>N</i> -terminus				CH ₃ : 2.07 (17.8) CH' ₃ : 1.93 (17.8) ^a
Cys ¹	8.35 7.62 ^b	5.01 ^b 5.03 ^{a, b}	3.31, 2.71 (28.0)	
Cys ²		5.38 (65.2)	3.48, 3.31 (37.2)	
Tyr ³		4.14 (52.9)	3.04, 2.74 (31.8)	δ : 6.78 (111.4); ϵ : 7.03 (126.3)
D-Trp ⁴	7.66	4.27 (50.5)	2.88, 2.95 (21.5)	δ 1: 6.86 (120.3); ϵ 1: 10.00; ϵ 3: 7.13 (115.2); ζ 2: 7.47 (107.8); ζ 3: 7.31 (114.0); η 2: 7.22 (117.8)
Lys ⁵		3.58	1.60	γ : 0.80, 0.69 (17.6); δ : 1.45 (22.4); ϵ : 2.76 (35.3); ζ : 7.45
Thr ⁶	7.66	4.36 (63.7)	4.47 (54.8)	γ 2: 1.28 (15.4)
Cys ⁷	8.18	4.64 ^b	3.66, 3.94	
Thr ⁸	8.00 8.11 ^b	4.32 (61.9) 4.42 ^{a, b}	4.02 (56.9)	γ 2: 1.28 (14.9) γ 2': 1.19 ^a

Chemical shifts were referenced relative to DSS and were measured at 25 °C. ¹H chemical shift values in ppm are listed with ¹³C values in parentheses. The chemical shifts are generally accurate to 0.02 ppm for ¹H and 0.1 ppm for ¹³C.

^a Chemical shifts of the minor conformations of the termini amino acids.

^b Chemical shifts of ¹³C $_{\alpha}$ could not be assigned due to overlapping resonances with H₂O in the ¹H-¹³C HSQC spectrum.

Table 4

Backbone-backbone NOEs of disulfide-cyclized TATE, previously synthesized [14], beta-sheet and distorted helical conformations

beta-sheet			distorted helical		
NOE	I	d	NOE	I	d
Phe ¹ αH-Cys ² HN	S	1.8–2.7 Å	Lys ⁵ αH-Thr ⁸ HN	W	1.8–6.0 Å
Cys ² αH-Tyr ³ HN	M	1.8–3.5 Å	Thr ⁶ HN-Thr ⁸ HN	W	1.8–6.0 Å
Thr ⁶ αH-Cys ⁷ HN	S	1.8–2.7 Å	Thr ⁶ αH-Thr ⁸ HN	W	1.8–6.0 Å
Thr ⁶ NH-Cys ⁷ HN	M	1.8–3.5 Å	Thr ⁶ HN-Cys ⁷ HN	M	1.8–3.5 Å
Cys ² αH-Cys ⁷ HN	M	1.8–3.5 Å	Cys ⁷ HN-Thr ⁸ HN	M	1.8–3.5 Å
Cys ² NH-Cys ⁷ HN	M	1.8–3.5 Å	Lys ⁵ αH-Thr ⁶ HN	W	1.8–6.0 Å
			Thr ⁶ αH-Cys ⁷ HN	S	1.8–2.7 Å
			Cys ⁷ αH-Thr ⁸ HN	M	1.8–3.5 Å

The strength of cross peak signal intensities (I) are presented as strong (S), medium (M), and weak (W). The distance (d) is listed as lower limit to upper limit.

Table 5

Backbone-backbone NOEs of disulfide-cyclized TATE, previously synthesized [14], beta turn

Turn			Disulfide Bridge		
NOE	I	d	NOE	I	d
Tyr ³ αH-Thr ⁶ HN	W	1.8–6.0 Å	dPhe ¹ αH-Cys ² NH	M	1.8–3.5 Å
dTrp ⁴ HN-Thr ⁶ HN	W	1.8–6.0 Å	Thr ⁶ αH-Cys ⁷ NH	M	1.8–3.5 Å
dTrp ⁴ αH-Thr ⁶ HN	W	1.8–6.0 Å	Cys ⁷ αH-Thr ⁸ NH	W	1.8–6.0 Å
dTrp ⁴ HN-Lys ⁵ HN	M	1.8–3.5 Å	Cys ⁷ NH-Thr ⁸ αH	W	1.8–6.0 Å
Lys ⁵ HN-Thr ⁶ HN	S	1.8–2.7 Å			
Tyr ³ αH-dTrp ⁴ HN	W	1.8–6.0 Å			
dTrp ⁴ αH-Lys ⁵ HN	M	1.8–3.5 Å			
Lys ⁵ αH-Thr ⁶ HN	M	1.8–3.5 Å			

The strength of cross peak signal intensities (I) are presented as strong (S), medium (M), and weak (W). The distance (d) is listed as lower limit to upper limit.

Table 6

Backbone-backbone NOEs of the beta-sheet conformation and turn of Re-cyclized TATE, previously synthesized [14].

Beta-Sheet			Turn		
NOE	I	d	NOE	I	d
dPhe ¹ αH-Cys ² NH	M	1.8–3.5 Å	dTrp ⁴ αH-Thr ⁶	S	1.8–2.7 Å
Thr ⁶ αH-Cys ⁷ NH	M	1.8–3.5 Å	dTrp ⁴ NH-Thr ⁶	S	1.8–2.7 Å
Cys ⁷ αH-Thr ⁸ NH	W	1.8–6.0 Å	dTrp ⁴ αH-Lys ⁵	M	1.8–3.5 Å
Cys ⁷ NH-Thr ⁸ αH	W	1.8–6.0 Å	Lys ⁵ NH-Thr ⁶	S	1.8–2.7 Å

The strength of cross peak signal intensities (I) are presented as strong (S), medium (M), and weak (W). The distance (d) is listed as lower limit to upper limit.