Generally Applicable, Convenient Solid-Phase Synthesis and Receptor Affinities of Octreotide Analogs

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Octreotide, an analogue of the hormone somatostatin, has applications as a therapeutic and imaging agent for somatostatin-positive tumors. We have developed a generally applicable, convenient stepwise solid-phase synthetic protocol for octreotide (D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-threoninol). [Cys(Acm)^2,D-Trp(Boc)^4,Lys(Boc)^5,Thr(*t*Bu)^6,Cys(Acm)^7,des(threoninol)]-octreotide was assembled by Fmoc solid-phase synthesis and the intramolecular disulfide bond formed by treatment of the resin-bound peptide with thallium trifluoroacetate [Tl(Tfa)$_3$]. Side-chain protection of Trp by the Boc group was found to preserve Trp integrity during Tl(Tfa)$_3$ treatment. The protected peptide was cleaved from the resin by aminolysis with threoninol and purified by semipreparative RP-HPLC. Isolated [D-Trp(Boc)^4,Lys(Boc)^5,Thr(*t*Bu)^6]octreotide had the correct molecular mass ([M + H]$^+$ = 1275 Da) and sequence and was obtained in 14% yield at >98% purity. [D-Trp(Boc)^4,Lys(Boc)^5,Thr(*t*Bu)^6]octreotide was utilized for the solution-phase synthesis of CPTA–D-Phe^1-octreotide, where CPTA is 4-[(1,4,8,11-tetraazacyclotetradec-1-yl)methyl]benzoic acid. Cyclic dianhydride of diethylenetriaminepentaacetic acid (DTPA) was coupled to a portion of the protected peptide-resin following disulfide bond formation. The DTPA-conjugated, side-chain-protected peptide was cleaved from the resin by aminolysis with threoninol, side-chain deprotected with trifluoroacetic acid, and purified by semipreparative RP-HPLC. The isolated DTPA–D-Phe^1-octreotide had the correct molecular mass ([M + H]$^+$ = 1395 Da) and was obtained in 5% yield at >90% purity. The efficiency of aminolysis was partially dependent upon the linkage between 4-(hydroxymethyl)phenoxy (HMP) handles and the resin and/or resin particle size. The somatostatin receptor binding affinities of synthetic DTPA–D-Phe^1-octreotide and CPTA–D-Phe^1-octreotide to AtT-20 mouse pituitary carcinoma cell membranes were examined by labeling with ^{111}In and ^{64}Cu, respectively, and performing Scatchard analyses. The dissociation constant (K_d) for our synthetic [^{111}In]DTPA–D-Phe^1-octreotide was 4.31 nM, which is comparable to a K_d = 5.57 nM obtained with commercially available DTPA–D-Phe^1-octreotide. The K_d for [^{64}Cu]CPTA–D-Phe^1-octreotide was 78.5 pM. On the basis of the criteria of molecular mass, RP-HPLC elution time, sequence analysis, and somatostatin receptor binding affinity, our synthetic octreotide is identical to commercially available octreotide. The aminolysis protocol used here has distinct advantages over either reductive cleavage or preformed linker methods described previously for the preparation of octreotide.

Introduction

Somatostatin, a cyclic peptide consisting of 14 amino acids, plays an inhibitory role in the hormonal regulation of at least three organ systems in humans: (i) the central nervous system, the hypothalamus, and pituitary gland, (ii) the gastrointestinal tract, and (iii) the exocrine and endocrine pancreas.[1–3] Somatostatin receptors have been characterized in these organ systems. Because of the "growth inhibitory" effect obtained in most tissues by the stimulation of somatostatin receptors, a large number of human tumors are also somatostatin receptor positive.[4] The pharmacological properties of somatostatin have generated an interest in the use of this peptide for therapeutic purposes. Much of the work has centered on neuroendocrine tumors, because of their somatostatin receptor concentrations;[5,6] however, other common types of neoplasms, such as gliomas,[7] lymphomas,[8] and breast cancers,[9] may also contain considerable numbers of somatostatin receptors. The ability to visualize somatostatin receptors *in vivo* for a large number of different tumor types using nuclear medicine will have a great impact on the biological characterization of cancers and will also be of value in making therapeutic decisions.

Somatostatin is unsuitable for *in vivo* use due to its very short biological half-life. An eight-amino acid peptide analogue of somatostatin, octreotide (**1**), has a longer half-life as well as inhibitory properties more potent than the native somatostatin.[10] Because of its growth hormone inhibitory effects, octreotide has been used as a clinical therapeutic agent to treat a wide variety of tumors which contain somatostatin receptors such as carcinoids and tumors of the pituitary and endocrine pancreas.[11] Octreotide has also been found

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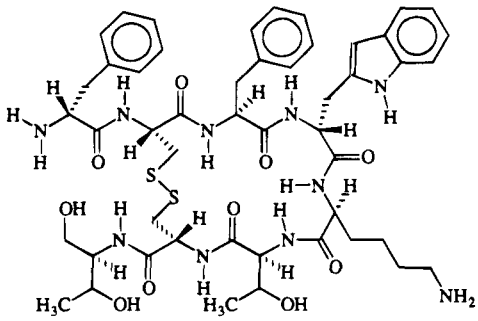

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to be clinically useful in treating the symptoms of acromegaly.[11,12] When conjugated to the chelate diethylenetriaminepentaacetic acid (DTPA) and labeled with ^{111}In, octreotide is a useful single-photon emission computed tomographic (SPECT) imaging agent of tumors containing somatostatin receptors.[13-15] Imaging studies involving [^{111}In]DTPA–D-Phe^1-octreotide in humans showed the activity to clear rapidly through the kidney, with >85% in the urine by 24 h.[16] Octreotide has also been conjugated with desferrioxamine (DFO) and labeled with either ^{67}Ga or ^{68}Ga.[17-19] The [^{68}Ga]-DFO–octreotide has been used for positron emission tomography (PET) studies of rat endocrine pancreatic tumors.[18,19] PET imaging with radiolabeled octreotide allows for the quantitation of somatostatin receptors and is therefore useful for predicting the clinical response of patients undergoing therapy with octreotide. To combine both PET imaging and therapeutic capabilities, octreotide has been conjugated with 4-[(1,4,8,11-tetraazacyclotetradec-1-yl)methyl]benzoic acid (CPTA) and labeled with ^{64}Cu.[20] ^{64}Cu is equally effective as ^{67}Cu in damaging DNA,[21] and hence ^{64}Cu-labeled octreotide analogs have potential as radiotherapeutics.

To evaluate the many possible octreotide conjugates as potential agents for cancer diagnosis and/or therapy, an adequate supply of precursor materials are required. At present, a general, convenient solid-phase protocol has not been described for the synthesis of octreotide, side-chain-protected octreotide, or chelator–octreotide conjugates. Although only eight residues, octreotide contains several structural features that contribute to a nontrivial synthesis. The formation of intramolecular disulfide bonds in the presence of Trp can be problematic.[22] In addition, there are no "standard" solid-phase protocols for the synthesis of *C*-terminal peptide alcohols. An ideal synthetic procedure would allow for the formation of the disulfide bond and incorporation of the chelator to occur while the peptide is resin-bound, taking advantage of the solid-phase method. Octreotide was synthesized initially by fragment condensation solution-phase procedures.[10,23,24] While synthetic "schemes" for these solution-phase procedures were presented, no extensive experimental methods or yields were given. Solid-phase synthesis of octreotide has been achieved previously by (i) synthesis and incorporation of peptide-resin "linkers" that produce a peptide alcohol upon cleavage and workup[25,26] or (ii) reductive cleavage of the peptide from the resin with $NaBH_4$/LiBr to produce a peptide alcohol.[27] Experimental details have been published for one of the linker methods,[25] where purified octreotide was isolated in a ~3.1% yield. However, neither the linker nor reductive cleavage methods are generally applicable. Utilizing linkers requires either multistep solution-phase preparation procedures or further modification of cleaved products to produce a *C*-terminal alcohol. Direct reductive cleavage of the resin-bound peptide to produce a peptide alcohol precludes on-resin disulfide bond formation and may be incompatible with on-resin incorporation of carboxylate-containing bifunctional chelators. On the basis of our own research experiences[28,29] and those of others,[30] we have examined mild aminolysis cleavage procedures for the solid-phase synthesis of side-chain-protected octreotide and DTPA–D-Phe^1-octreotide and compared the efficiency of this protocol with reductive cleavage. We have utilized the side-chain-protected octreotide for the solution-phase synthesis of CPTA–D-Phe^1-octreotide. The somatostatin receptor binding affinity of our synthetic material is also compared to the commercially available product.

Syntheses

The resin-bound peptide sequences, assembled by 9-fluorenylmethoxycarbonyl (Fmoc) chemistry, were (**I**) D-Phe-Cys(Acm)-Phe-D-Trp-Lys(Boc)-Thr(*t*Bu)-Cys(Acm)-Thr(*t*Bu)-HMP resin [where Acm is acetamidomethyl and HMP is 4-(hydroxymethyl)phenoxy], (**II**) D-Phe-Cys(Acm)-Phe-D-Trp-Lys(Boc)-Thr(*t*Bu)-Cys(Acm)-Thr(*t*Bu)-HMPB resin [where HMPB is 4-(4-(hydroxymethyl)-3-methoxyphenoxy)butyric acid], (**III**) D-Phe-Cys(Acm)-Phe-D-Trp-Lys(Boc)-Thr(*t*Bu)-Cys(Acm)-HMP resin, and (**IV**) D-Phe-Cys(Acm)-Phe-D-Trp(Boc)-Lys(Boc)-Thr(*t*Bu)-Cys(Acm)-HMP resin. In addition, peptide **IV** was synthesized on two different Fmoc-Cys(Acm)-HMP resins, one prepared by "preformed handle" strategy[22] (**IVa**) and one prepared by "handle-resin" strategy[22] (**IVb**).

Reductive Cleavage. The direct reductive cleavage of a resin-bound peptide to produce a peptide alcohol was initially achieved with $LiBH_4$.[31] Treatment of peptide-resin **I** under the same conditions produced a heterogeneous crude product. Edman degradation analysis indicated that reductive cleavage of peptide bonds had occurred, as several amino acids from the octreotide sequence were identified in the first cycle of analysis. Reductive cleavage of peptide bonds by $LiBH_4$ has been documented previously.[32] Milder reductive cleavage conditions, such as the use of $NaBH_4$ instead of $LiBH_4$,[33] were then examined. Treatment of peptide-resin **I** with $NaBH_4$/LiBr (1:1) in ethanol–tetrahydrofuran (1:6)[27] for 18 h gave a major peak on analytical reversed-phase high-performance liquid chromatography (RP-HPLC) eluting at 28.8 min (Figure 1). ESMS analysis of the isolated peak (~8% overall yield) suggested the structure D-Phe-Cys(Acm)-Phe-D-Trp-Lys(Boc)-Thr(*t*Bu)-Cys(Acm)-Thr(ol), as $[M + H]^+$ = 1375 Da (calculated = 1375.0 Da). Reductive treatment of peptide-resin **II**, which utilizes the more acid-labile HMPB linker,[34] resulted in increased product heterogeneity and decreased yield. Incorporation of the cyclic dianhydride of DTPA (cDTPAA) onto peptide-resin **I** followed by $NaBH_4$/LiBr treatment gave a heterogenous RP-HPLC elution profile with no apparent major peak.

Disulfide Bond Formation. Solid-phase methods have been described for direct disulfide bond formation from Cys(Acm) residues by I_2, $Tl(Tfa)_3$, or $Hg(OAc)_2$ treatments.[35,36] Peptide-resin **III** or **IVa** was treated by each of these reagents and cleavage performed with H_2O–TFA (1:19). RP-HPLC analysis of the I_2-treated

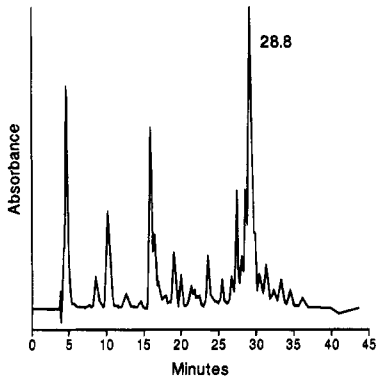

Figure 1. RP-HPLC elution profile of products following "mild" reductive cleavage of peptide-resin **I**. The elution gradient was 25–80% B in 40 min at a flow rate of 1.0 mL/min, where A was 0.1% TFA in H_2O and B was 0.1% TFA in acetonitrile. Other conditions are given under Experimental Section. D-Phe-Cys(Acm)-Phe-D-Trp-Lys(Boc)-Thr(*t*Bu)-Cys-(Acm)-Thr(ol) eluted at 28.8 min.

peptide gave two distinct product peaks, but attempted workup resulted in a subsequent heterogeneous elution product profile. RP-HPLC analysis of the $Hg(OAc)_2$-treated peptide gave two prominent peaks eluting at 17.7–17.8 and 18.3–18.4 min. RP-HPLC purification and ESMS analysis of these two products indicated that the early eluting peak was the desired peptide ($[M + H]^+$ = 933 Da, calculated = 933.4 Da) while the later eluting peak was the desired peptide plus Hg^{2+} ($[M + H]^+$ = 1132 Da, calculated = 1132.0 Da). Extended or repeated 2-mercaptoethanol washes of the peptide-resin following $Hg(OAc)_2$ treatment did not affect the quantity of Hg^{2+}-modified peptide produced. RP-HPLC analysis of the 1 h $Tl(Tfa)_3$-treated peptide-resin **IVa** gave two prominent peaks eluting at 17.1 and 17.6–17.8 min (Figure 2). RP-HPLC purification and ESMS analysis of these two products indicated that the later eluting peak was the desired oxidized peptide ($[M + H]^+$ = 933 Da, calculated = 933.4 Da) while the early eluting peak was one mass unit higher than the desired peptide ($[M + H]^+$ = 934 Da). Extended or repeated $Tl(Tfa)_3$ treatments did not improve the ratio of these peaks. Disulfide bond formation by $Tl(Tfa)_3$ was repeated with peptide-resin **IVb** for 1.0 h. RP-HPLC of the peptide showed primarily one product eluting at 23.8 min (Figure 2), which had $[M + H]^+$ = 933 Da.

Aminolysis. Initial aminolysis experiments were performed on peptide-resin **III** to determine optimal conditions for threoninol [Thr(ol)] cleavage. A 24 h treatment with Thr(ol) at 55 °C gave a single peak by RP-HPLC, eluting at 25.2 min (Figure 3). Increased time and/or temperature decreased the homogeneity of the product. Identical treatments of peptide-resins **III** and **IVb** with Thr(ol) for 22 h at 55 °C resulted in cleavage yields of 18 and 88%, respectively.

Protected and DTPA-Octreotide. The best results from the disulfide and aminolysis experiments were combined to generate an optimal synthetic protocol for [D-Trp(Boc)^4,Lys(Boc)^5,Thr(*t*Bu)^6]octreotide and DTPA–D-Phe^1-octreotide (Scheme 1). Following peptide assembly, the *N*-terminal Fmoc group was removed with piperidine–DMF (1:1) and the peptide-resin washed with DMF. Disulfide bond formation of peptide-resin **IVb** proceeded with $Tl(Tfa)_3$ for 1 h. The peptide-resin was washed three times each with DMF and methanol. Some resin was removed for DTPA addition (see below).

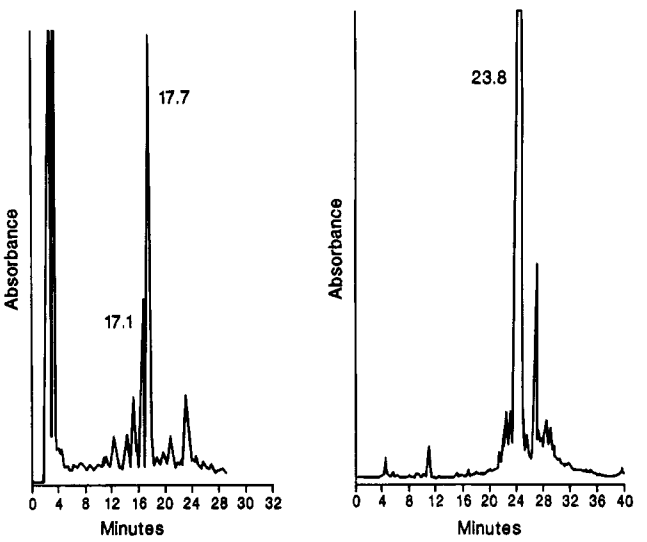

Figure 2. RP-HPLC elution profile of products following $Tl(Tfa)_3$ treatment and TFA cleavage of peptide-resin (left) **IVa** and (right) **IVb**. For analysis of disulfide bond formation on peptide-resin **IVa**, the elution gradient was 25–35% B in 30 min at a flow rate of 1.5 mL/min, where A was 0.1% HFBA in H_2O and B was 0.1% HFBA in acetonitrile. For analysis of disulfide bond formation on peptide-resin **IVb**, the elution gradient was 10–60% B in 40 min at a flow rate of 1.0 mL/min, where A was 0.1% TFA in H_2O and B was 0.1% TFA in acetonitrile. Other conditions are given under Experimental Section. DesThr(ol)-octreotide eluted at either (left) 17.7 min or (right) 23.8 min.

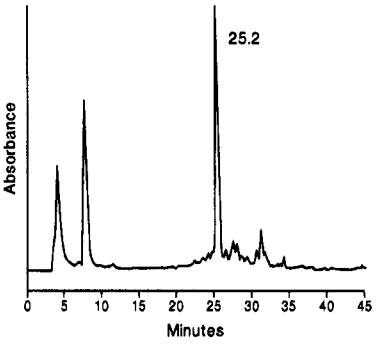

Figure 3. RP-HPLC elution profile of products following aminolysis of peptide-resin **III**. The elution gradient was 25–80% B in 40 min at a flow rate of 1.0 mL/min, where A was 0.1% TFA in H_2O and B was 0.1% TFA in acetonitrile. Other conditions are given under Experimental Section. [Cys-(Acm)^2,Lys(Boc)^5,Thr(*t*Bu)^6,Cys(Acm)^7]octreotide eluted at 25.2 min.

Aminolysis proceeded by treating 44 mg of the peptide-resin with 75 mg of Thr(ol) in 500 μL of DMF for 22 h at 55 °C. The resin was filtered and washed two times with 250 μL of DMF and one time with 250 μL of methanol. The filtrate was diluted with 200 μL of 2-propanol–H_2O (1:1) containing 0.1% TFA and purified by RP-HPLC using a gradient of 30–50% B in 40 min at a flow rate of 3.0 mL/min, where A = 0.1% TFA in H_2O and B = 0.1% TFA in 2-propanol. RP-HPLC gave two major products, one eluting at 16.9 min and one eluting at 23.3 min (Figure 4). ESMS analysis of the isolated peaks showed both the early eluting product ($[M + H]^+$ = 1273 Da) and the later eluting product ($[M + H]^+$ = 1275 Da) to have masses comparable to the desired product (calculated $[M + H]^+$ = 1275 Da). Following TFA treatment to remove side-chain protecting groups, the early eluting product ($[M + H]^+$ = 1019

Scheme 1

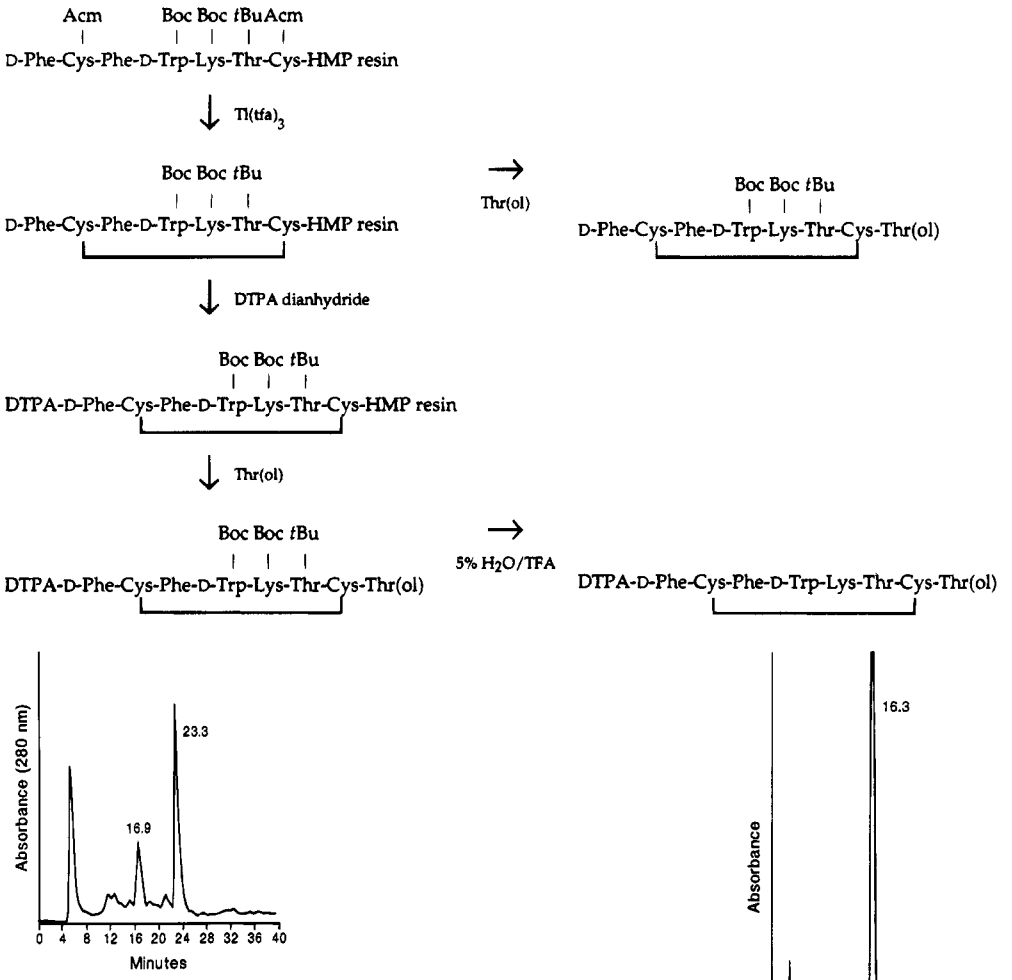

Figure 4. RP-HPLC elution profile of crude [D-Trp(Boc)^4,Lys(Boc)^5,Thr(tBu)^6]octreotide. The elution gradient was 40–60% B in 40 min at a flow rate of 0.6 mL/min, where A was 0.1% TFA in H_2O and B was 0.1% TFA in 2-propanol. Other conditions are given under Experimental Section. [D-Trp(Boc)^4,Lys(Boc)^5,Thr(tBu)^6]octreotide eluted at 23.3 min.

Figure 5. RP-HPLC elution profile of purified [D-Trp(Boc)^4,Lys(Boc)^5,Thr(tBu)^6]octreotide. The elution gradient was 20–30% B in 40 min at a flow rate of 1.5 mL/min, where A was 0.1% TFA in H_2O and B was 0.1% TFA in acetonitrile. Other conditions are given under Experimental Section. [D-Trp(Boc)^4,Lys(Boc)^5,Thr(tBu)^6]octreotide eluted at 16.3 min.

Da) and the later eluting product ($[M + H]^+$ = 1018 Da) both still had masses comparable to the desired product (calculated $[M + H]^+$ = 1019 Da). Coelution with an octreotide standard revealed the later eluting product to be the desired peptide. Yield of isolated [D-Trp(Boc)^4,Lys(Boc)^5,Thr(tBu)^6]octreotide (prior to TFA treatment; Figure 5) was 2.75 mg (14%). Edman degradation analysis gave the sequence Phe-Cys-Phe-Trp-Lys-Thr-Cys. (The *C*-terminal Thr(ol) cannot be analyzed by Edman chemistry, as it cannot form a phenylthiohydantoin derivative.)

CPTA–D-Phe^1-octreotide was prepared by reacting 467 μg (1.4 μmol) of CPTA, 216 μg (1.6 μmol) of HOBt, and 182 μg (1.4 μmol) of DIC with 200 μg (0.14 μmol) of [D-Trp(Boc)^4,Lys(Boc)^5,Thr(tBu)^6]octreotide (TFA salt) in 100 μL of DMF for 3 h. CPTA–D-Phe^1-[D-Trp(Boc)^4,Lys(Boc)^5,Thr(tBu)^6]octreotide was purified by RP-HPLC using a gradient of 45–75% B in 60 min, where A = 0.1% TFA in H_2O and B = 0.1% TFA in acetonitrile. Side-chain deprotection proceeded with H_2O–TFA (1:19) for 5 min. CPTA–D-Phe^1-octreotide was 98% pure by RP-HPLC. ESMS analysis gave $[M + H]^+$ = 1335 Da (calculated $[M + H]^+$ = 1335 Da). The overall yield for the preparation of CPTA–D-Phe^1-octreotide from Fmoc-Cys(Acm)-HMP resin was 4.1%.

DTPA was coupled to peptide-resin **IVb** *after* disulfide bond formation by treating 50 mg peptide-resin with cDTPAA (4 equiv) and *N,N*-diisopropylethylamine (20 equiv) in DMF for 0.75 h. In cases where the reaction was not complete by ninhydrin analysis,[22] DTPA was recoupled. Excess anhydride was quenched by the addition of 50 μL of H_2O. The resin was washed three times each with DMF and methanol. Aminolysis of 45 mg of peptide-resin with 175 mg of Thr(ol) in 350 μL of DMF was performed for 22 h at 55 °C. The resin was filtered and washed two times with 250 μL of DMF and one time with 250 μL of methanol. The filtrate was concentrated *in vacuo*, and the product was treated with 1.5 mL of H_2O–TFA (1:19) for 5 min, diluted with 2.2 mL of H_2O, and concentrated *in vacuo*. The crude product was dissolved in 2-propanol–H_2O (1:9) containing 0.1% TFA and purified by RP-HPLC using a gradient of 10–40% B in 60 min at a flow rate of 3.0 mL/min, where A = 0.1% TFA in H_2O and B = 0.1% TFA in 2-propanol. The product peak eluting at 22.9

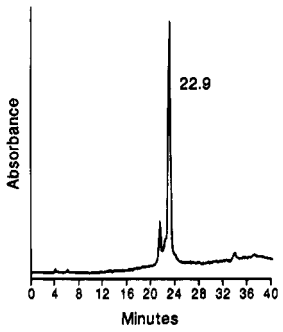

Figure 6. RP-HPLC elution profile of purified DTPA–D-Phe^1-octreotide. The elution gradient was 10–60% B in 40 min at a flow rate of 1.0 mL/min, where A was 0.1% TFA in H_2O and B was 0.1% TFA in acetonitrile. Other conditions are given under Experimental Section. DTPA–D-Phe^1-octreotide eluted at 22.9 min.

min (Figure 6) gave $[M + H]^+ = 1395$ Da by ESMS, corresponding to DTPA–D-Phe^1-octreotide (calculated $[M + H]^+ = 1395$ Da). The yield of isolated DTPA–D-Phe^1-octreotide was 0.8 mg (5%). Using the same protocol with 189 mg of peptide-resin **IVa** resulted in a yield of 0.12 mg (0.2%).

Receptor Binding Activities

The binding affinities of the synthetic DTPA–D-Phe^1-octreotide labeled with ^{111}In and CPTA–D-Phe^1-octreotide labeled with ^{64}Cu were evaluated using membranes from mouse anterior pituitary adenoma (AtT-20) cells. AtT-20 cells contain a high concentration of somatostatin receptors[37] and have thus been used extensively in the study of somatostatin receptor binding and in the characterization of the receptor itself.[38] Somatostatin has been shown to inhibit both adrenocorticotropin secretion and cAMP formation in AtT-20 cells.[39] Scatchard analyses of [^{111}In]DTPA–D-Phe^1-octreotide and [^{64}Cu]CPTA–D-Phe^1-octreotide binding to AtT-20 cell membranes were linear, and the dissociation constants (K_d) were 4.31 nM (53% error) and 78.5 pM (31% error), respectively. Scatchard analysis of [^{111}In]DTPA–D-Phe^1-octreotide prepared from commercially available DTPA–D-Phe^1-octreotide (Octreoscan) was also linear with $K_d = 5.57$ nM (17% error).

Discussion

The solid-phase synthesis of octreotide had several potential pitfalls that could have reduced peptide assembly and cleavage efficiencies and/or resulted in deletereous side reactions. Potential problems included (i) racemization of the *C*-terminal Cys residue, (ii) inefficient disulfide bond formation on-resin, (iii) modification of Trp during disulfide bond formation, and (iv) incomplete peptide-resin cleavage by aminolysis. In regard to (i), peptide-resins **III** and **IV** were assembled with a *C*-terminal esterified Cys(Acm) residue. Although *C*-terminal esterified Cys residues are racemized by repeated base deprotection treatments during Fmoc solid-phase synthesis,[40] the extent of racemization induced by either piperidine or DBU during octreotide assembly is low. The required 1.75 h treatment of the *C*-terminal Cys(Acm) residue with 20% piperidine or 2% DBU would result in acceptable 2.4 and 1.2% conversion to D-Cys, respectively.[40,41] The extent of racemization of esterified Cys(Acm) is less than either Cys(S*t*Bu) or Cys(Trt),[40] hence the preference for Acm side-chain protection of Cys. The use of a more sterically hindered linker-resin, which was shown to reduce Cys racemization during somatostatin syntheses,[42] would not be effective here due to potential difficulties during aminolysis (see discussion below).

Potential problems (ii) and (iii) were examined by attempting on-resin disulfide bond formation with either I_2, $Hg(OAc)_2$, or $Tl(Tfa)_3$. Treatment of Cys(Acm) residues with I_2, which produced a heterogeneous product upon workup, has been shown previously to cause side reactions at Trp, resulting in Trp-2′-thioethers and β-3-oxindolylalanine.[43,44] Treatment of the Cys(Acm) residues with $Hg(OAc)_2$ showed a residual Hg^{2+} ion present. The Hg^{2+} ion was probably not bound by Cys but instead had modified Trp, as (i) repeated 2-mercaptoethanol treatments did not remove the Hg^{2+} and (ii) the molecular mass of the product corresponded to a peptide with a disulfide bond present and two protons displaced. Hg(OAc) can modify Trp during $Hg(OAc)_2$ treatment of Cys(Acm) residues.[45] Direct disulfide formation with $Tl(Tfa)_3$ resulted in the cleanest product. Initial studies with $Tl(Tfa)_3$ claimed that Trp needed to be side-chain protected during peptide treatment this reagent.[46] Our results indicated that side-chain protection of Trp by the Boc group (peptide-resin **IV**) was sufficient to ensure preservation of Trp integrity during $Tl(Tfa)_3$ treatment.

Peptide-resin cleavage by aminolysis was examined as a function of time, temperature, and resin. Prior work had shown the advantages of DMF over methanol or dichloromethane for cleavage of peptide-resins by aminolysis.[28,29] Peptide homogeneity was best preserved by a single, 22 h aminolysis reaction at 55 °C. Higher yields of desired products were obtained using peptide-resin **IVb** compared with peptide-resin **III** or **IVa**, suggesting that a careful consideration of resin particle size and the chemical linkages between the handle and resin is required. The first Fmoc-Cys(Acm)-HMP resin (**III** or **IVa**) was prepared using a "preformed handle" strategy, *i.e.*, synthesizing an activated ester of Fmoc-Cys(Acm)-HMP and acylating it to aminomethyl resin,[47] creating an $-OCH_2CONHCH_2-$ linkage between the HMP handle and the resin. The second Fmoc-Cys(Acm)-HMP resin (**IVb**) was prepared by esterification of Fmoc-Cys(Acm) to HMP resin with 2,6-dichlorobenzoyl chloride.[48] HMP resin was prepared as described by Wang,[49] which results in a $-OCH_2-$ linkage between the HMP handle and the resin. The additional amide linkage in the first resin resulted in a lower yield of DTPA–D-Phe^1-octreotide, possibly due to susceptibility of cleavage by aminolysis. In addition, the larger particle size of peptide-resin **III** or **IVa** (100–200 mesh) compared with **IVb** (200–400 mesh) may have contributed to the different yields.

Peptide alcohols may be produced by mild reductive cleavage of peptide-resins, thus avoiding aminolysis reactions. The peptide D-Phe-Cys(Acm)-Phe-D-Trp-Lys-(Boc)-Thr(*t*Bu)-Cys(Acm)-Thr(ol), obtained by $NaBH_4$/LiBr cleavage of peptide-resin **I**, was isolated in a yield of 8%. Other researchers using the same mild reductive cleavage conditions produced Boc-D-Phe-Cys(Acm)-Phe-D-Trp-Lys(Boc)-Thr(*t*Bu)-Cys(Acm)-Thr(ol) with an RP-HPLC purity of 45%.[27] Incorporation of a chelator onto the peptide-resin followed by reductive cleavage resulted in a heterogeneous product mixture, suggesting that reductive cleavage methods may compromise the integ-

rity of some chelators. Since both chelator incorporation and disulfide bond formation are best performed after reductive cleavage, the aminolysis approach is clearly the more convenient method.

A higher isolated peptide yield was obtained for [D-Trp(Boc)^4,Lys(Boc)^5,Thr(*t*Bu)^6]octreotide (14%) as compared with DTPA–D-Phe^1-octreotide (5%). This result is most likely due to use of cDTPAA.[50] The yield of DTPA–D-Phe^1-octreotide could be improved by using the preformed DTPA HOBt ester[51] instead of cDTPAA. The HOBt ester ensures that only monomeric DTPA–D-Phe^1-octreotide and not dimeric DTPA–(octreotide)$_2$ is formed. Other chelators which may have more desirable properties than DTPA, such as DFO or CPTA, are acylated using monomeric species instead of anhydrides, and thus should be efficiently incorporated by our protocol. The isolation of [D-Trp(Boc)^4,Lys(Boc)^5,Thr(*t*Bu)^6]octreotide allows for a variety of solution-phase preparations of octreotide derivatives, as demonstrated by the synthesis of CPTA–D-Phe^1-octreotide.

ESMS characterization of [D-Trp(Boc)^4,Lys(Boc)^5,Thr(*t*Bu)^6]octreotide, CPTA–D-Phe^1-octreotide, and DTPA–D-Phe^1-octreotide indicated that all purified products had the desired molecular masses. In addition, [D-Trp(Boc)^4,Lys(Boc)^5,Thr(*t*Bu)^6]octreotide gave the correct sequence Phe-Cys-Phe-Trp-Lys-Thr-Cys by Edman degradation analysis, and TFA treatment of this peptide resulted in a product that coeluted with commercially available octreotide on RP-HPLC. The K_d value obtained for [^{111}In]DTPA–D-Phe^1-octreotide (4.31 nM) is very close to that obtained for [^{111}In]DTPA–D-Phe^1-octreotide derived from commercial sources (5.57 nM). On the basis of these criteria, this synthetic octreotide is identical to commercially available octreotide. [^{64}Cu]-CPTA–D-Phe^1-octreotide, which has potential application as both an imaging agent and a therapeutic,[20] had a K_d value (78.5 pM) even lower than [^{111}In]DTPA–D-Phe^1-octreotide.

The solid-phase protocol developed here is currently the most generally applicable synthetic procedure described for octreotide. Comparison of aminolysis to reductive cleavage showed that the latter method produced deleterious side reactions and did not permit on-resin disulfide bond formation. The third alternative for octreotide synthesis, that of utilizing peptide alcohol producing linkers compatible with Fmoc solid-phase chemistry,[26,52] can require difficult linker preparation. For example, one method is based upon synthesizing Tfa-Thr(ol)-acetal from Tfa-Thr(ol) and *p*-formylphenoxyacetic acid and coupling this product to aminomethyl resin.[26] The Tfa group is removed by $NaBH_4$ in methanol–tetrahydrofuran, and the peptide is assembled by standard Fmoc chemistry. This linker method did permit on-resin intramolecular disulfide bond formation (via deprotection of Cys *tert*-butyl thiol groups with tributylphosphine and hydrogen peroxide-catalyzed cyclization). However, an overall comparison of the three methods for octreotide synthesis indicates that the aminolysis protocol is the most easily applied, allows for incorporation of a variety of chelators, and results in a highly homogeneous product. The *p*-formylphenoxyacetic acid procedure would be more feasible if Fmoc-Thr(*t*Bu)(ol) could be used instead of Tfa-Thr(ol). It has recently been reported that treating *N*-urethane protected amino acid *N*-carboxy anhydrides (UNCAs) with $NaBH_4$ produces β-amino alcohols.[53] These results suggest that Fmoc-Thr(*t*Bu)(ol) could be produced by reduction of commercially available Fmoc-Thr(*t*Bu)-NCA. Although offering a reasonable alternative, this still would not be as generally applicable as the solid-phase protocol described here.

Experimental Section

General Aspects. *N,N*-Diisopropylethylamine, 1-methyl-2-pyrrolidinone, piperidine, ProSpin sample preparation cartridges, and trifluoroacetic acid (TFA) were from Applied Biosystems, Inc. (Foster City, CA); acetonitrile, *N,N*-dimethylformamide (DMF), dichloromethane, mercuric acetate [$Hg(OAc)_2$], and 4-(dimethylamino)pyridine (DMAP) from Fisher; 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), *N,N'*-diisopropylcarbodiimide (DIPCDI), dithiothreitol, DTPA dianhydride, iodoacetamide, $LiBH_4$, LiBr, $NaBH_4$, 10% Pd/C, tetrahydrofuran (THF), and thallium trifluoroacetate [$Tl(Tfa)_3$] from Aldrich; heptafluorobutyric acid (HFBA) from Pierce; octreotide (Sandostatin) from DePaul Professional Drugs (Bridgeton, MO); DTPA–D-Phe^1-octreotide (Octreoscan) and $^{111}InCl_3$ from Mallinckrodt Medical (St. Louis, MO); absolute ethanol from Barnes Hospital (St. Louis, MO); preloaded HMP resins from either Millipore Corp. (Bedford, MA) [Fmoc-Thr(*t*Bu)-HMP resin, sub. level = 0.32 mmol/g; Fmoc-Cys(Acm)-HMP resin, sub. level = 0.36 mmol/g] or Novabiochem (La Jolla, CA) [Fmoc-Cys(Acm)-HMP resin, sub. level = 0.55 mmol/g]; HMPB resin (sub. level = 0.57 mmol/g) and 1-hydroxybenzotriazole (HOBt) from Novabiochem; threoninol(Bzl)·HCl from Advanced ChemTech (Louisville, KY); $^{64}CuCl_2$ from the University of Missouri Research Reactor (MURR); and 2-(1*H*-benzotriazol-1–yl)-1,1,3,3-tetramethyluronium hexafluorophosphate from Richelieu Biotechnologies (St.-Hyacinthe, Quebec). Resins from Millipore were 100–200 mesh, 1% divinylbenzene cross-linked polystyrene, while those from Novabiochem were 200–400 mesh, 1% divinylbenzene cross-linked polystyrene. Fmoc-L- and -D-amino acids were from Novabiochem or Millipore. CPTA was synthesized as described.[54]

Thr(ol) was prepared by a modification of the method of Anwer and Spatola.[55] Threoninol(Bzl)·HCl and ammonium formate (2 equiv) were dissolved in methanol–acetic acid (1:1). The solution was poured onto 10% Pd/C, and the reaction was monitored by thin-layer chromatography (methanol–$CHCl_3$, 1:1). When debenzylation was complete, the reaction mixture was filtered through Celite and concentrated *in vacuo* to a pale yellow oil. Thr(ol) was loaded onto BioRad AG50W-X8 ion-exchange resin (H^+ form), rinsed with H_2O, and eluted with 14% aqueous ammonia. Fractions positive to ninhydrin were pooled and concentrated to a pale yellow oil. Yield was typically on the order of 80%. Chemical shifts (recorded on a Varian Gemini-300 300 MHz spectrometer, in ppm relative to internal tetramethysilane): ^{1}H-NMR (DMSO-d_6) 3.58 (m, 1 H), 3.49 (dd, 2 H, J_d = 10 Hz, J_{dd} = 6 Hz), 3.33 (dd, 1 H, J_d = 10 Hz, J_{dd} = 6 Hz), 1.12 (d, 3 H, J = 6 Hz); ^{13}C-NMR (DMSO-d_6) 67.9, 64.6, 60.0, 21.6.

Preparation of Fmoc-Thr(*t*Bu)-HMPB Resin. Fmoc-Thr(*t*Bu) (1.78 g, 4.48 mmol), HOBt (0.61 g, 3.98 mmol), and DMAP (0.054 g, 0.44 mmol) were dissolved in 20 mL of DMF and added to 2.0 g of HMPB resin (1.14 mmol). After shaking for 5 min, 0.714 mL of DIPCDI (4.56 mmol) was added, and esterification proceeded for 4 h. The resin was washed two times with DMF and one time with DCM and stored under vacuum overnight. The substitution level of Fmoc-Thr(*t*Bu)-HMPB resin was determined by spectrophotometric analysis[22] to be 0.19 mmol/g.

Peptide Synthesis. Peptides were synthesized using Fmoc chemistry as described previously.[56,57] The resin-bound sequences were (**I**) D-Phe-Cys(Acm)-Phe-D-Trp-Lys(Boc)-Thr(*t*Bu)-Cys(Acm)-Thr(*t*Bu)-HMP resin, (**II**) D-Phe-Cys(Acm)-Phe-D-Trp-Lys(Boc)-Thr(*t*Bu)-Cys(Acm)-Thr(*t*Bu)-HMPB resin, (**III**) D-Phe-Cys(Acm)-Phe-D-Trp-Lys(Boc)-Thr(*t*Bu)-Cys(Acm)-HMP resin, and (**IV**) D-Phe-Cys(Acm)-Phe-D-Trp(Boc)-Lys(Boc)-Thr(*t*Bu)-Cys(Acm)-HMP resin. In addition, peptide **IV** was synthesized on two different Fmoc-Cys(Acm)-HMP resins,

one prepared by "preformed handle" strategy (sub. level = 0.36 mmol/g) (**IVa**) and one prepared by "handle-resin" strategy (sub. level = 0.55 mmol/g) (**IVb**). Edman degradation analysis confirmed the sequence and indicated <2% cumulative preview of each peptide, establishing a high efficiency of assembly.

Peptide-resin **I** was reductively cleaved under "strong" and "mild" conditions, while peptide-resin **II** was reductively cleaved under "mild" conditions. "Strong" conditions were 3 equiv of 2 M $LiBH_4$ in THF for 1 h, while "mild" conditions[27] were 12 equiv of 0.24 M $NaBH_4$/LiBr (1:1) in ethanol–THF (1:6) for 18 h. THF was freshly distilled prior to use. Reactions were quenched by the addition of methanol. Products were recovered by semipreparative RP-HPLC.

The efficiency of on-resin disulfide bond formation was examined by reacting peptide-resin **III** and/or **IV** with $Hg(OAc)_2$, $Tl(Tfa)_3$, or I_2 in DMF. Treatment with $Hg(OAc)_2$ (0.1 M; 4 equiv) proceeded for 1 or 4 h, followed by washings with 10 equiv of 2-mercaptoethanol one time for 18 h or twice for 1.5 and 24 h. $Tl(Tfa)_3$ (0.4 M; 1.5 equiv) treatments were either one time for 1 or 24 h or twice for 1 and 51 h. Treatment with I_2 (0.06 M, 10 equiv) proceeded for 1 h at 0 °C. After each disulfide bond formation treatment, the peptide-resin was washed with DMF, CCl_4, CH_2Cl_2, and methanol and cleaved with H_2O–TFA (1:19). Aminolysis of 30 mg of peptide-resin **III** or **IV** proceeded with 4 μL of Thr(ol) and 500 μL of DMF for (i) 22–24 h at room temperature, (ii) 22–24 h at 55 °C, (iii) 24 h at room temperature plus 18 h at 45–50 °C, (iv) 48 h at 50–60 °C, or (v) 72 h at 45–50 °C. Aminolysis reactions were performed in tightly sealed reactivials with magnetic stirring.

Peptide Purification and Characterization. Analytical and semipreparative RP-HPLC was performed on a Waters 600E multisolvent delivery system equipped with either a Vydac 218TP54 C_{18} column (300 Å pore size, 10 μm particle size, 250 × 4.6 mm) or a Vydac 218TP1010 C_{18} column (300 Å pore size, 10 μm particle size, 250 × 10 mm), a Spectra Physics chromjet integrator, and a Rheodyne 7125 injector. Detection was at 280 nm with a Waters 484 tunable absorbance detector. For analysis of disulfide bond formation on peptide-resins **III** or **IVa**, the elution gradient was 25–35% B in 30 min at a flow rate of 1.5 mL/min, where A was 0.1% HFBA in H_2O and B was 0.1% HFBA in acetonitrile. For analysis of disulfide bond formation on peptide-resin **IVb**, the elution gradient was 10–60% B in 40 min at a flow rate of 1.0 mL/min, where A was 0.1% TFA in H_2O and B was 0.1% TFA in acetonitrile. For analysis of the reductive cleavage of peptide-resin **I** and aminolysis reaction of peptide-resin **III**, the elution gradient was 25–80% B in 40 min at a flow rate of 1.0 mL/min, where A was 0.1% TFA in H_2O and B was 0.1% TFA in acetonitrile. For analysis of the aminolysis reaction of peptide-resin **IVb**, the elution gradient was 40–60% B in 40 min at a flow rate of 0.6 mL/min, where A was 0.1% TFA in H_2O and B was 0.1% TFA in 2-propanol. For analysis of chelator acylation, the elution gradient was 10–60% B in 40 min at a flow rate of 1.0 mL/min, where A was 0.1% TFA in H_2O and B was 0.1% TFA in acetonitrile.

Edman degradation analysis was performed on an Applied Biosystems 477A Protein Sequencer/120A Analyzer as described previously for "embedded" (noncovalent) sequencing.[58] Prior to sequencing, purified [D-Trp(Boc)^4,Lys(Boc)^5,Thr(tBu)^6]-octreotide (1 mg, 0.78 μmol) was dissolved in a solution containing 0.5 mL of acetonitrile and 0.5 mL of 0.1 M Tris plus 6 M guanidine–HCl. Following the addition of 11 mg (0.071 mmol) dithiothreitol, the solution was incubated at 40 °C for 4 h. Iodoacetamide (23 mg, 0.12 mmol) was added, and the reaction proceeded for 15 min at room temperature in the dark. The reaction mixture was diluted with 1.0 mL of H_2O–acetonitrile (1:1) and filtered under centrifugation with a ProSpin sample preparation cartridge. The PDVF membrane from ProSpin was used directly for sequence analysis. Fulvene–piperidine concentrations (301 nM) were determined with a Beckman DU-70 spectrophotometer. Electrospray mass spectrometric (ESMS) analyses were performed either as described previously[58] or with a Vestec 201 mass spectrometer.

^{111}In Labeling of DTPA–D-Phe^1-octreotide, ^{64}Cu Labeling of CPTA–D-Phe^1-octreotide, and Receptor Binding. DTPA–D-Phe^1-octreotide (5 μg) was dissolved in 100 μL of 0.1 M ammonium citrate, pH 4.0. ^{111}In citrate was generated from $^{111}InCl_3$ as described.[59] ^{111}In citrate (5.38 mCi in 0.1 M ammonium citrate, pH 4.0) was added to the DTPA–D-Phe^1-octreotide solution and incubated at room temperature for 1 h. After loading onto a C_{18} SepPak and washing with citrate buffer, [^{111}In]DTPA–D-Phe^1-octreotide was recovered by elution with 1 mL of ethanol. CPTA–D-Phe^1-octreotide was labeled with ^{64}Cu acetate (0.1 M ammonium acetate, pH 5.5) and purified by a similar procedure. ^{64}Cu acetate was generated from $^{64}CuCl_2$ as described.[59]

The somatostatin receptor binding affinities of [^{111}In]DTPA–D-Phe^1-octreotide and [^{64}Cu]CPTA–D-Phe^1-octreotide were determined by Scatchard analysis of binding assays. A preparation of cell membranes was made from AtT-20 cells (courtesy of Dr. David Parkinson, Department of Cell Biology and Physiology, Washington University) by brief sonication and centrifugation at 13 000 rpm. Cell membranes were resuspended in receptor buffer.[60] Equal volumes of membranes were added to varying concentrations of [^{111}In]DTPA–D-Phe^1-octreotide or [^{64}Cu]CPTA–D-Phe^1-octreotide (±1000-fold excess of Sandostatin as blocker) in triplicate ranging over a concentration range of 2 orders of magnitude. Samples were incubated for 2 h at 25 °C and then centrifuged to separate the pellet from the buffer. The pellet was washed twice, and both fractions were counted. Data were plotted as nM [^{111}In]DTPA–D-Phe^1-octreotide or [^{64}Cu]CPTA–D-Phe^1-octreotide specifically bound versus the bound/free ratio and K_d values calculated using the program LIGAND.[61]

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