Lanthionine−Somatostatin Analogs: Synthesis, Characterization, Biological Activity, and Enzymatic Stability Studies

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A series of cyclic somatostatin analogs containing a lanthionine bridge have been subjected to studies of structure−activity relationships. A direct synthesis of the thioether bridged analog (**1**) of sandostatin (SMS 201,995) and several lanthionine hexa-, hepta-, and octapeptides was carried out by using the method of cyclization on an oxime resin (PCOR) followed by condensation reactions in solution. The structures of the target peptides were analyzed by liquid secondary ion mass spectrometry (LSIMS) and subjected to high-energy collision-induced dissociation (CID) studies after opening of the peptide ring by proteolytic cleavage. The biological activities of these compounds have been evaluated by assaying their inhibitory potencies for the release of growth hormone (GH) from primary cultures of rat anterior pituitary cells, as well as by their binding affinities to cloned somatostatin receptors (SSTR1-5). The structural modification of sandostatin by introducing a lanthionine bridge resulted in a significantly increased receptor binding selectivity. The lanthionine octapeptide with C-terminal Thr-ol (**1**) showed similar high affinity for rat SSTR5 compared to somatostatin[1-14] and sandostatin. However, it exhibits about 50 times weaker binding affinity for mSSTR2b than sandostatin. Similarly, the lanthionine octapeptide with the C-terminal Thr-NH_2 residue (**2**) has higher affinity for rSSTR5 than for mSSTR2B. Both peptides (compounds **1** and **2**) have much lower potencies for inhibition of growth hormone secretion than sandostatin. This is consistent with their affinities to SSTR2, the receptor which is believed to be linked to the inhibition of growth hormone release by somatostatin and its analogs. The metabolic stability of lanthionine−sandostatin and sandostatin have been studied in rat brain homogenates. Although both compounds have a high stability toward enzymatic degradation, the lanthionine analog has a 2.4 times longer half-life than sandostatin. The main metabolites of both compounds have been isolated and identified by using an *in vivo* technique (cerebral microdialysis) and mass spectrometry.

Introduction

Somatostatin, a key regulatory hormone, was isolated from bovine hypothalami in 1973.[1] Biological studies revealed that native somatostatin occurs in two biologically active forms, a tetradecapeptide somatostatin-14 (Figure 1) and a 28-residue peptide, somatostatin-28. Both are derived from the same polypeptide precursor: prosomatostatin.[2]

Somatostatin (both the 14-mer and the 28-mer peptides) exhibits diverse physiological activities.[3] In the central nervous system, somatostatin has a neurotransmitter function, modulates locomotor activity, and has cognitive functions. At a cellular level, it inhibits the adenylylcyclase activity and calcium ion conductance and potentiates K^+ currents. Through these functions somatostatin acts as an endocrine hormone, as a paracrine substance, and as an autocrine secretagogue.[4] The actions of somatostatin are mediated *via* membrane bound receptors. The heterogeneity of its biological functions together with its short half-life has challenged many researchers to develop more stable compounds. This has led to studies to identify structure−activity relationships of peptides at the somatostatin receptors which resulted in the discovery of five somatostatin receptor subtypes.[5,6] These receptor subtypes [SSTR1-5] have been cloned and characterized.[6,7] Their specific functional roles are under extensive investigation. Recent pharmacological studies have demonstrated a definite correlation between the receptor affinity for mSSTR2b and the inhibition of growth hormone secretion by somatostatin analogs.[7] The receptor SSTR5 has been proposed to have a limited role in growth hormone regulation but instead may have a selective function in controlling insulin secretion.[8,9] In drug research, it is a key issue to minimize side effects by developing highly potent and selective drug molecules. In our studies, we set the goal to design and synthesize new and selective somatostatin analogs.

Extensive structure−activity studies are important in order to discover possible therapeutic applications of

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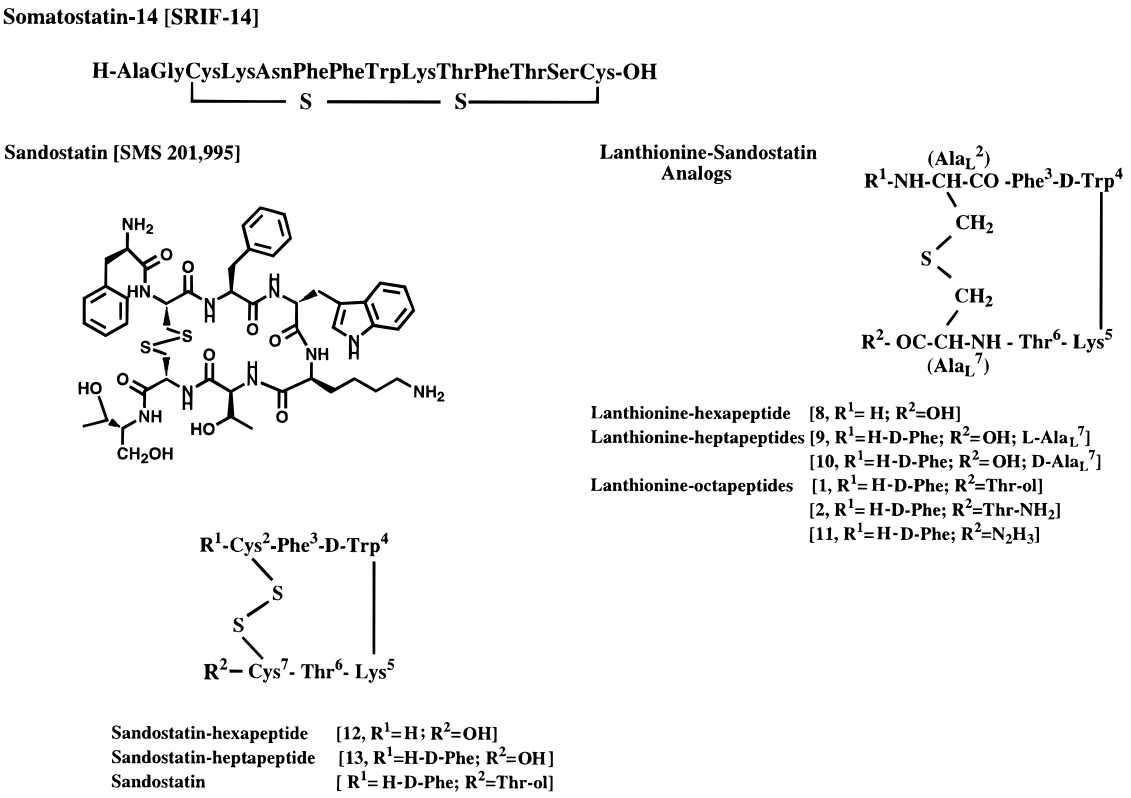


Figure 1. Structures of natural somatostatin, synthetic sandostatin, and lanthionine–sandostatin analogs.

somatostatin analogs. Structural modifications of native somatostatin such as the incorporation of D-amino acids, peptidomimetics, and cyclization have led to the discovery of analogs with extended half-life and increased biological activities.[10,11] Veber et al. suggested that the amino acid sequence Phe^7-Trp^8-Lys^9-Thr^{10} (the superscript numbers here denote the relative positions of the amino acids in the native somatostatin), kept in β-II' turn conformation, it is necessary for biological activity.[11] This hypothesis led Veber and his colleagues[12] to the design and synthesis of the highly active cyclic hexapeptide somatostatin analog c[Pro^6-D-Trp^8-Lys^9-Thr^{10}-Phe^{11}].

If either Phe^7 or Phe^{11} is replaced by an Ala residue in the parent compound, the *in vitro* activity for the inhibition of growth hormone release drops by about 90%.[13] Furthermore, the free ϵ-amino group of a Lys residue has been shown to be necessary for eliciting bioactivity.[14] In addition, the D-Trp at position 8 is highly sensitive to substitution and modification. Replacement of D-Trp with D-Phe causes a drop of 2 orders of magnitude in the inhibition of growth hormone release. Substitution of Pro^6 and Thr^{10} affects the biological activity to a much lower extent.[15] Therefore, only residues Phe^7, D-Trp^8, Lys^9, and Phe^{11} are thought to be primarily responsible for somatostatin activity.

Conformational analysis of the bioactive analogs containing backbone modifications such as retroinverso modification[16] and the completely retro backbone[17] has indicated that the peptide backbone is not directly involved in binding, but serves mainly as a scaffold which allows the side chains to adopt the necessary pharmacophore spatial arrangement.

The amino acid sequence Phe-D-Trp-Lys-Thr can be stabilized in the bioactive conformation if a disulfide bridge is used in place of the Pro-Phe bridge. Stepwise modification of the cyclic hexapeptide c[Cys-Phe-D-Trp-Lys-Thr-Cys] led to the synthesis of the highly active somatostatin analog, sandostatin (SMS 201, 995), H-D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-Thr-ol (Figure 1).[18] On the basis of conformational analysis of sandostatin, the authors suggested that the disulfide bridge might not be sufficient to constrain the cyclic backbone in the proposed bioactive conformation.[19] They supposed that Thr-ol, as the C-terminal residue and D-Phe, as the N-terminal residue might have additional stabilizing effect on the active conformation through the formation of an antiparallel β-sheet-like structure.[20]

However, the exocyclic residues (D-Phe and Thr-ol) have only moderate conformational stability which results in a decreased stability of the bioactive conformation of sandostatin. In order to introduce further conformational constraints into the ring skeleton, we have designed a series of somatostatin analogs in which the disulfide bridge has been replaced by a lanthionine monosulfide bridge (Figure 1).[21] The goal of the present work focuses on structural modifications of sandostatin to obtain an improved pharmacological profile. These modifications led to the development of compounds with improved selectivity at SSTR5.

Results

Peptide Design and Synthesis. In the design of our lanthionine–somatostatin analogs, our strategy was to constrain the backbone conformation of the biologically highly active sandostatin without changing the spatial arrangements of the pharmacophore groups (Phe^7, Trp^8, and Lys^9) and to enhance the stability of sandostatin.

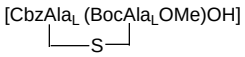
Molecular modeling studies of sandostatin and lanthionine–sandostatin molecules were carried out to compare the possible and preferred conformations of the

disulfide and the lanthionine-bridged structures in the cyclic hexapeptides (Figure 1). These investigations indicated that the removal of one sulfur atom from the 20-membered cyclic hexapeptide will not alter the orientation of the pharmacophore groups. Furthermore, we believed that the reduced ring size might lead to a more stable conformation.

In our synthetic strategy, the fully protected lanthionine hexapeptide (**3**) was the key intermediate for the preparation of the hepta- and octapeptides through addition of the exocyclic residues (D-Phe[1], Thr[8]NH_2, or Thr[8](Bzl)-ol). Recently, our laboratory has developed and applied new synthetic routes for preparation of lanthionine peptides.[22,23]

For the preparation of the lanthionine hexapeptide (**3**), we extruded a sulfur atom from an existing disulfide bridge by using the HMPA reagent.[24] We have previously used this methodology for the synthesis of lanthionine enkephalins.[25] However, in our present work, these reactions resulted in extremely low yields (<5%). Under the conditions used, the formyl protecting group was cleaved from the indole ring (determined by HPLC and mass spectrometry analyses) faster than the extrusion of the sulfur atom occurred. These results support our previous experience with "sulfur-removal" reactions which require not only fully protected peptides but the use of base-stable protecting groups.

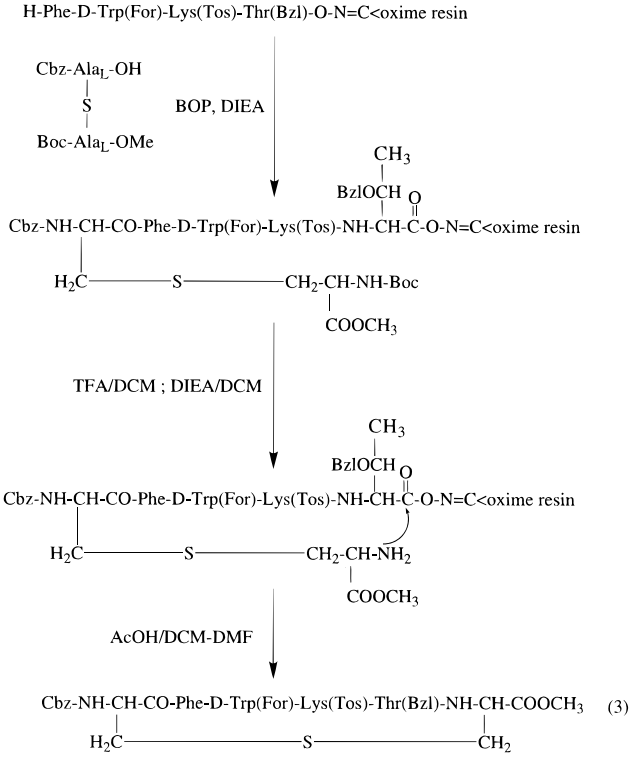
A novel synthetic route has been developed in our laboratory for the synthesis of lanthionine peptides. This method is based upon the use of an appropriately protected lanthionine amino acid building block



and the method of peptide cyclization on an oxime resin (PCOR).[26,27] [The Cbz and Boc protecting groups can be selectively removed.] The synthesis was initiated by the preparation of BocThr(Bzl)-oxime resin (substitution level: 0.26 mmol/g, determined by picric acid titration[28]). The peptide chain was assembled by using Boc chemistry and BOP activation method (Scheme 1). Finally, the protected lanthionine residue (**4**) was coupled to the peptide chain by using 3-fold molar excess of the amino acid and BOP reagent. The reaction was completed within 1 h according to the Kaiser test.[29] The Boc group was then cleaved from the lanthionine residue followed by the peptide cyclization reaction with acetic acid catalysis. The reaction was carried out for 24 h according to the general procedure of the PCOR method.[26] The product (**3**) was obtained after DMF/water and DMF/ether precipitations in a 65% yield based upon the starting material, BocThr(Bzl) oxime resin.

Condensations of the cyclic hexapeptide (**3**) with the C- and the N-terminal exocyclic amino acids were carried out in solution (Scheme 2). The benzyloxycarbonyl group was removed from the N-terminus with HBr/acetic acid treatment (which also cleaved the Bzl protecting groups). A 5-fold excess of Cbz-D-Phe[1]-OH activated with EDC/HOBt was used to form the peptide bond (yield 86%). In the next step, the heptapeptide methyl ester was converted to the hydrazide (**5**, yield 60.9%). The azide (prepared by butyl nitrite/HCl treatment) was allowed to react with a 6-fold excess of threonine-amide or threonine(Bzl)-ol (yields 97.2% and

Scheme 1. Synthesis of a Lanthionine−Sandostatin Intermediate on an Oxime Resin



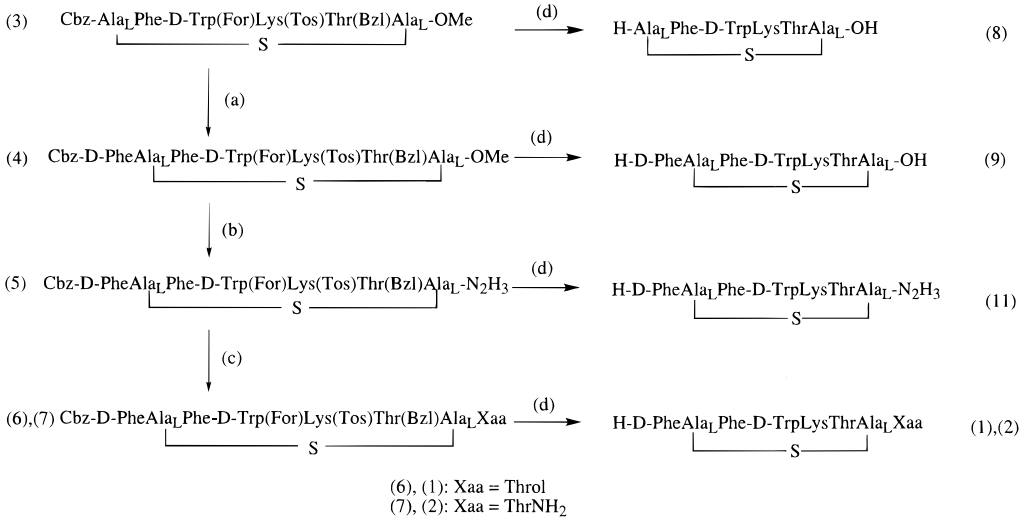
93.7%), respectively. The final deprotection of the hexa- and heptapeptides was carried out in two steps: first the methyl ester group was hydrolyzed with 0.05 N NaOH/HMPA−water (1/1, v/v) at 10 °C for 1 h, and then the remaining protecting groups were removed by Na/NH_3 treatment. The crude products were subjected to Sephadex G-10 gel filtration and HPLC purification. Two peaks in the HPLC spectrum of the crude heptamer showed identical UV and mass spectra. Consequently, the two compounds must be diastereomers in a ratio of 3:2. The diastereomers were separable by HPLC. Two dimensional NMR spectroscopy was used to make the configurational assingments at residue 7. The main product was the desired molecule (**9**) with the L configuration at residue 7. The heptamer **9** exhibits an $H_\alpha{}^2H_\alpha{}^7$ NOE cross peak, which was present in all the other analogs with an L configuration in the tail unit of the lanthionine residue. The heptamer **10** does not show this NOE cross peak. These results allowed us to assign the D configuration to the chiral center $C_\alpha{}^7$ in the carboxyl terminal lanthionine unit for heptamer **10**.

The hexa- and heptapeptide analogs of sandostatin with disulfide bridges (**12** and **13**) were assembled in an ABI model 433A automatic peptide synthesizer using standard solid phase procedure (Scheme 3). An HMPB resin served as solid phase support, and Fmoc chemistry and BOP/HOBt activation were used in the syntheses. The disulfide bridge formation was carried out by iodine oxidation on the resin.[30] Finally, Reagent-K was used to cleave the peptides from the resin with simultaneous cleavage of the protecting groups. The target disulfide-bridged peptides were obtained by reversed-phase HPLC purification.

NMR and Mass Spectrometry

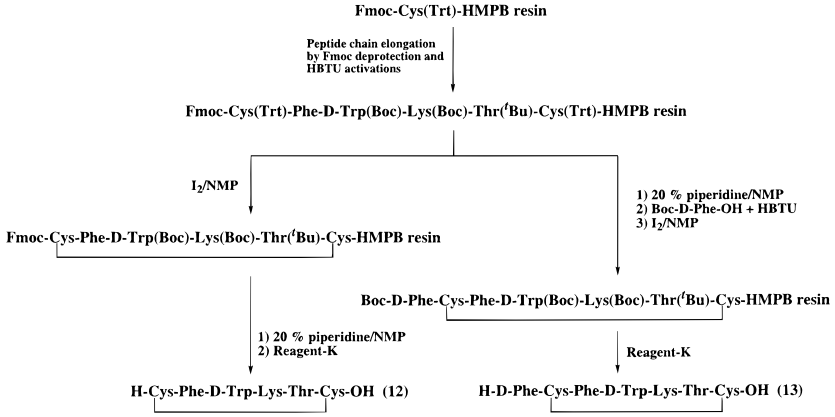
FAB mass spectrometry was used to verify the identities of all intermediates and final compounds. The

Scheme 2. Synthesis of Lanthionine−Sandostatin Derivatives[a]



[a] (a) (1) HBr/AcOH, (2) Cbz-D-PheOH + EDC + HOBt; (b) N_2H_4; (c) (1) ^{t}BuNO$_2$ + HCl, (2) H-Thr(Bzl)-ol or H-Thr-NH$_2$; (d) (1) NaOH, (2) Na/NH$_3$.

Scheme 3



structural details of each compound were investigated by ^{1}H NMR spectroscopy. The assignment of every signal in the 500 MHz NMR spectra was possible with the use of two-dimensional HOHAHA, COSY, and ROESY experiments. The intramolecular NOE effects between the α-protons of the lanthionine residue was used to determine the chirality of the two somatostatin heptamers (**9**, **10**) separated by HPLC. The ^{1}H NMR data of intermediates are listed in the Experimental Section. Detailed NMR spectra and conformational analysis of the target compounds are presented in an accompanying paper.[44]

In the last decade, mass spectrometry has also developed as a method of choice for characterizing synthetic peptides.[31] For studying the molecular structure of our unprotected hexa-, hepta-, and octapeptides, all samples were subjected to liquid secondary ion mass spectrometry (LSIMS) analysis. Each compound produced the expected molecular ion [MH$^+$] as listed in Table 1. Although high-energy collision-induced dissociation (CID) analysis[32,33] of the cyclic peptides provides some information about their structure, the open chain structures were expected to show more extensive fragmentation.[34] Thus, the cyclic peptides were subjected to proteolytic digestion with chymotrypsin. The chymotryptic digestion resulted in ring opening (a molecular mass increase of 18 Da, see Table 1). The newly formed species were also subjected to high-energy CID analysis. Fragmentation observed in these experiments confirmed the amino acid sequence of the peptides and the location of the sulfide linkage. Figure 2 shows the high-energy CID fragmentation of the chymotrypsin-digested sulfide-bridged heptapeptide. The immonium and related ions at *m*/*z* 74; 84, 101, and 129; 120; 130, 159, and 170 indicated the presence of Thr, Lys, Phe, and Trp residues, respectively.[35] Ions y_1, y_2 and y_3 at *m*/*z* 503, 604, and 732, respectively, formed *via* peptide bond cleavages with charge retention at the C-terminus, reveal the amino acid sequence for the longer chain (nomenclature according to ref 36). The corresponding N-terminal ions, b_2 and b_3, were detected at *m*/*z* 315 and 416, respectively. Loss of Phe residues from both ends of the shorter chain were observed yielding y_2* ion at 771 and b_2* ion at *m*/*z* 753. Some of the most abundant fragments resulted from double cleavages. They appear at masses corresponding to sequential amino acid losses from y_2* ion, at *m*/*z* 585 (Trp), 457 (Lys), and 356 (Thr), respectively (labeled with asterisks in Figure 2).

A bond cleavage at the sulfur atom, with charge retention on the more basic (i.e., Lys-containing) peptide chain yields the ion at *m*/*z* 536 (indicated with an arrow in Figure 2). The analogous high-energy CID fragmentation of the disulfide-bridged heptapeptide was observed. All fragment ions containing the cross-linkage are shifted by 32 Da because of the presence of the

Table 1. Molecular Masses Measured for the Lanthionine and Disulfide-Bridged Peptides before and after Enzymatic Ring Opening

compound	[MH$^+$] theoretical	[MH$^+$] measured	[MH$^+$] after chymotryptic digestion
H-Ala$_L$-Phe-D-Trp-Lys-Thr-Ala$_L$-OH (**8**)	753.34	752.8	752.8 not cleaved
H-D-Phe-Ala$_L$-Phe-D-Trp-Lys-Thr-Ala$_L$-OH (**9**)	900.41	900.1	918.5
H-D-Phe-Ala$_L$-Phe-D-Trp-Lys-Thr-Ala$_L$-Thr-ol (**1**)	987.48	987.1	1005.5
H-D-Phe-Ala$_L$-Phe-D-Trp-Lys-Thr-Ala$_L$-Thr-NH$_2$ (**2**)	1000.47	1000.5	1018.5
H-Cys-Phe-D-Trp-Lys-Thr-Cys-OH (**12**)	785.31	785.3	785.3 not cleaved
H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-OH (**13**)	932.38	932.4	950.5
H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol	1,019.45	1,019.7	1,037.6

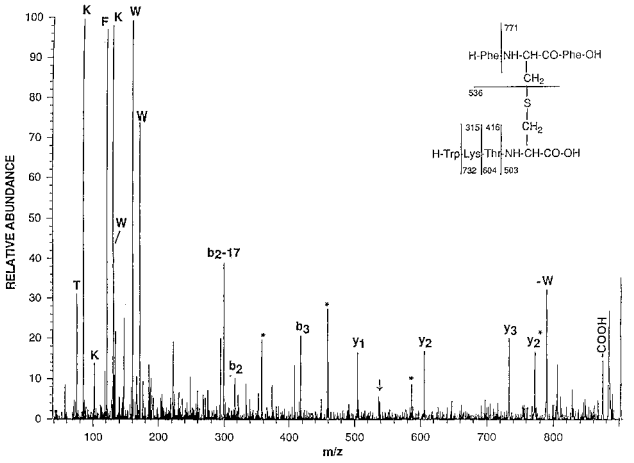


Figure 2. High-energy CID spectrum of the chymotrypsin-digested lanthionine heptapeptide. ([MH$^+$] at *m*/*z* 918.5). The immonium and related low mass ions are labeled with the one letter code of the corresponding amino acid. Peptide sequence ions are labeled according to the nomenclature.[36] Fragment y_2* corresponds to the loss of the N-terminal D-Phe residue from the shorter chain. Ions labeled with asterisks are formed via double bond cleavages and can be described as sequential amino acid losses from y_2* ion. The fragment formed *via* cleavage at the sulfur atom is indicated with an arrow.

additional sulfur atom. Thus, ions y_1, y_2, and y_3 are at *m*/*z* 535, 636, and 764, respectively. Similarly, fragment y_2* is detected at *m*/*z* 803, while ions formed *via* sequential amino acid or peptide segment loss from y_2* ion at *m*/*z* 617 (Trp), 489 (TrpLys), and 388 (TrpLysThr) (Figure 1S in the Supporting Information). Cleavage in the disulfide bridge yield ions at *m*/*z* 567 and at *m*/*z* 536.

Biological Results. The native somatostatin hormone has a broad variety of biological activities as we briefly reviewed in the Introduction. In our study, we examined the ability of the synthetic peptides to inhibit the release of growth hormone *in vitro*. The compounds were also tested for specific binding to the six cloned somatostatin receptors [hSSTR1, mSTTR2b, mSSTR3, hSSTR4, rSSTR5, and hSSTR5 (h = human, m = mouse, r = rat)] expressed in CHO cell membranes.[37,38] The results are summarized in Table 2. Somatostatin-14 and its analog, sandostatin, are used as reference materials in the evaluation of the biological potencies of the lanthionine−somatostatin analogs. In earlier studies we have demonstrated that the lanthionine analog of somatostatin-14 has a similar binding affinity to mSSTR2b as the natural disulfide-bridged peptide.[39]

The biological results for the new lanthionine−sandostatin analogs showed that only the octamers (**1** and **2**) inhibit GH release with EC_{50} values in the nanomolar range, but they are approximately 100-fold less potent than somatostatin. These results are in good agreement with the binding data of these compounds measured on SSTR1-5 subtypes. Compounds **1** and **2** bind to mSSTR2b with 13−16 nM affinities. These affinities are approximately 50-fold weaker than somatostatin-14. There are no clear pharmacological differences between rodent and human SSTR1, SSTR2, SSTR3, and SSTR4. Only SSTR5 exhibits species variation in the receptor pharmacology.[40] Compounds **8**−**10** did not bind to SSTR2b and did not affect GH release. Our lanthionine hexa- and the diastereomeric heptamers showed no binding to this receptor type. It is worthwhile noting that the lanthionine hexapeptide (**8**) and the heptapeptide (**9**) containing L-Ala$_L$[7] have weak activity, but only for rSSTR5, a receptor not associated with inhibition of growth hormone secretion. The disulfide-bridged heptapeptide (**13**) is equally potent on mSSTR2b, rSSTR5, and hSSTR5.

The two lanthionine octapeptides potently bind to both rat and human SSTR5. Lanthionine−sandostatin has similar affinity for rSSTR5 as sandostatin, but it has approximately 50-fold lower affinity for mSSTR2b than somatostatin. By comparing the ratios of IC_{50} values for mSSTR2b/rSSTR5, the receptor binding assays of the disulfide- and monosulfide-bridged octapeptide alcohols show that the lanthionine analog is about 40 times more selective for rSSTR5. For human SSTR5, the lanthionine octapeptide amide has affinity similar to that of the threoninol analog.

The lanthionine analogs showed no affinity ($IC_{50} > 1$ μM) for hSSTR1, mSSTR3, and hSSTR4, while somatostatin-14 is active in subnanomolar level at these receptors. In contrast to lanthionine−sandostatin, sandostatin has high affinity for mSSTR3 and hSSTR4 ($IC_{50} = 3$ nM). These results indicate that the introduction of the lanthionine bridge into sandostatin provides an altered receptor binding profile, namely a higher selectivity for SSTR5.

Biodegradation Studies *in Vitro* and *in Vivo*

Biostability is a key issue in peptide drug development. For centrally active peptides, degradation in brain tissue takes on special significance. Cerebral breakdown may limit the duration of action and also can change pharmacological profiles. Thus, biological

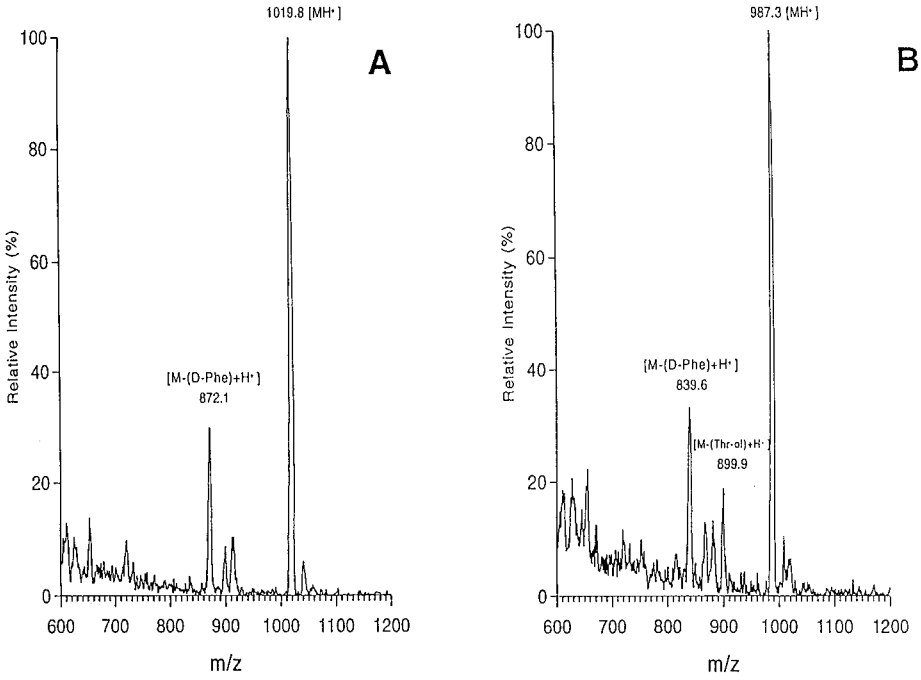


Figure 3. Molecular ion [MH^+] regions of the ESI mass spectra form dialysates collected during infusion of sandostatin (A) and lanthionine−sandostatin (B) into rat brain (striatum).

Table 2. Potencies of Somatostatin Analogs To Inhibit Radioligand Binding to Cloned Somatostatin Receptors and To Inhibit GH Release from Primary Cultures of Male Rat Anterior Pituary Cells

		IC_{50} (nM)[a]					Ic_{50} ratio	inhibn of GH
no.	compound	mSSTR2b	mSSTR3	hSSTR4	rSSTR5	hSSTR5	SSTR2b/rSSTR5	Release IC_{50} (nM)
	somatostatin, SRIF[1-14]	0.28	0.08	0.08	0.86		0.33	0.52 ± 0.7
	sandostatin, SMS-201,995	0.28	3	3	1.04	0.77	0.27	0.52 ± 0.7
1	H-D-Phe-Ala$_L$-Phe-D-Trp-Lys-Thr-Ala$_L$-Thr-ol (S bridge)	13.13	>1000	>1000	1.29	4.17	10.2	48 ± 1.8
2	H-D-Phe-Ala$_L$-Phe-D-Trp-Lys-Thr-Ala$_L$-Thr-NH_2 (S bridge)	16.60	>1000	>1000	5.50	4.92	3.02	70 ± 13
8	H-Ala$_L$-Phe-D-Trp-Lys-Thr-Ala$_L$-OH (S bridge)	>1000	>1000	>1000	500	N/D	>2	>1000
9	H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-OH (S-S bridge)	>1000	>1000	>1000	500	N/D	>2	>1000
13	H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-OH (S-S bridge)	13.62	>1000	>1000	10.63	10.95	0.75	N/D

[a] Data are expressed as IC_{50} (nM) ± SEM where available; $n = 5$ replicate determinations. None of the compounds, except natural somatostatin, showed biological activity on hSSTR1.

action may be mediated through conversion to metabolites.[41] Biostability in brain tissue is, therefore, an important property of newly developed, centrally acting peptide analogs such as those discussed in this paper. Rat brain homogenate[42] is commonly used to evaluate the possible fate of molecules in the CNS *in vitro*, because both soluble and membrane-bound peptidases are present in these preparations. An *in vivo* technique utilizing cerebral microdialysis and mass spectrometry has also been developed to identify extracellular metabolites of peptides in the central nervous system.[43]

The enzymatic degradation rates of lanthionine−sandostatin (**1**) and sandostatin, as reference material, have been determined by incubating the peptides with rat brain homogenates at 37 °C and pH 7.4 for 90 min. The process of degradation was followed by determining the remaining peptide concentration by HPLC analysis. Concentration−time profiles were analyzed by exponential fitting, assuming a pseudo-first-order degradation process. Half-lives (Table 3) were calculated from the rate constants (k) as 0.693/k.

Self-proteolytic products of excised brain tissue gave high "background" in chromatographic analysis which prevented us from locating and identifying CNS metabolites from these rather stable peptides *in vitro*. Therefore, an *in vivo* technique involving the introduction of the peptides into the brain *via* a microdialysis probe fitted with a semipermeable polymer membrane (20 000 Da cutoff) was utilized.[43] In this technique, the drug enters from the probe by diffusion (driven by the concentration gradient between the inside of the probe and the extracellular space of the brain), and metabolites are formed in the living tissue. The metabolic fragments, in turn, distribute themselves into the probe side solution free of interference by *in vitro* artifacts and are collected in the dialysate by the continuous perfu-

Table 3. Stability of Lanthionine−Sandostatin (**1**) Compared with SMS-201,995 against Degradation by Rat Brain Homogenate

compound	k (1/min)	$t_{1/2}$ (h)	standard deviation (1/min)	regression coefficient R^2 (1/min)
lanthionine−sandostatin	5.5×10^{-4}	30.3	4.5×10^{-5}	0.99379
sandostatin	1.3×10^{-3}	12.5	4.8×1^{-4}	0.99795

sion of the microdialysis device. The identities of the *in vivo* metabolites are then determined by ESI mass spectrometric analysis (Figure 3).

The main degradation product of sandostatin corresponds to the heptapeptide formed by enzymatic cleavage of the N-terminal D-Phe [protonated molecule, MH^+, found at *m/z* 872.1 in the ESI mass spectrum, expected *m/z* 872.1]. The lanthionine–sandostatin also showed the loss of the N-terminal D-Phe (MH^+, found at *m/z* 839.6, expected *m/z* 840.0) as a major metabolic pathway. The loss of the C-terminal Thr-ol (MH^+, found at *m/z* 899.9, expected *m/z* 900.1) in the ESI mass spectrum was a minor metabolite. No other metabolites by cleavage of peptide bonds inside of the disulfide-bridged and the lanthionine cyclopeptide were detected.

Discussion

Our synthetic efforts resulted in a series of lanthionine-bridged sandostatin analogs by utilizing our recently developed lanthionine ring formation method on an oxime resin. This method was used to prepare the fully protected lanthionine-bridged hexapeptide (**3**) that served as synthetic precursor for all lanthionine–sandostatin analogs. Although this strategy requires a preprepared protected lanthionine unit, the peptide synthesis and the cyclization reaction proceed smoothly in high yields. The protecting groups used (Boc, Bzl, For, Tos) are generally applicable in other PCOR syntheses.[39] Chromatographic (HPLC analysis/purity angle on a Water's Milleneum system) and spectroscopic (1D and 2D-NMR, LSIMS, and CID studies) analyses of all compounds established their purities and structures. A comparison of conformationally relevant NMR parameters of the molecules was used to determine the chirality of the Ala_L^7 residue in a diastereomeric pair (**9** and **10**). The D-Ala_L^7 residue in compound **10** is a consequence of a partial racemization which occurred during the ester hydrolysis of compound **4**. Low levels of racemization (<2%) were observed during a similar ester hydrolysis on the hexapeptide (**3**).

Earlier studies including the synthesis of a series of sandostatin analogs with different C-terminal residues[18] have shown that, for the strongest somatostatin-like activity, threonine derivatives must be incorporated as the C-terminal residue (Thr-NH_2 or Thr-ol). Both the C- and the N-terminal residues play a significant role in the manifestation of high biological activity. Lack of the threonine alcoholic hydroxyl group resulted in reduced activity. In our study, we designed and synthesized compounds in which the peptide ring is more constrained than in sandostatin. This smaller ring size should increase the conformational stability of the cyclic backbone. However, the biological tests showed no or very little activity for the lanthionine heptapeptides (**9**, **10**, and **11**) and both mono- and disulfide-bridged hexapeptides (**8** and **12**). These findings suggest that the two exocyclic residues (D-Phe^1 and Thr^8-ol or amide) might have specific function in receptor binding. The disulfide-bridged heptapeptide (**13**) shows significant activity but no selectivity on mSSTR2b and r/hSSTR5. In addition, the type of the C-terminal end group slightly affects the binding potency. For example, the disulfide-bridged hexapeptide acid (**12**) does not have somatostatin-like activity; however, its C-terminal amide analog has weak activity according to the literature.[18]

Biological potencies, conformational properties, and physiological behavior of lanthionine analogs of sandostatin have been investigated and compared with the properties of sandostatin. High biological potencies were expected since the Phe^3-D-Trp^4-Lys^5-Thr^6 tetrapeptide, which is considered as the active region, remained intact and a preferred type II' β-turn backbone conformation in this tetrapeptide region exists in both molecules. Our conformational studies[44] established these similarities. In accordance with the conformational changes, significant biological alterations were found in *in vitro* bioactivity, receptor binding, and binding selectivity. The two lanthionine octapeptides (**1** and **2**) have reduced affinities for the mSSTR2b receptor (about 1/50 of the binding affinity [IC_{50}] of sandostatin). However, the affinity of compound **1** for rSSTR5 is similar to those of somatostatin-14 and sandostatin. High receptor selectivity is important in order to reduce side effects. The $[IC_{50}]_{mSSTR2b}/[IC_{50}]_{rSSTR5}$ affinity ratio determined for sandostatin and compounds **1** and **2** are 0.3, 10.2, and 2.9, respectively. The lanthionine–sandostatinamide (**2**) has a 25% weaker binding to mSSTR2b than lanthionine–sandostatin (**1**), which leads to differences in their *in vitro* inhibitory activity in the release of GH from primary cultures of rat anterior pituitary cells. It is important to highlight that the two octapeptide lanthionine–sandostatin analogs have an influence on GH release–inhibition about 2 orders of magnitude weaker than sandostatin. The lower affinities for mSSTR2b and lower inhibitory activity on GH release confirms the suggestion[7] that this somatostatin receptor subtype is involved in GH secretion.

The metabolic stability of sandostatin was expected to be increased by introducing a lanthionine bridge in the place of the disulfide moiety. Our previous studies on lanthionine opioids indicated that the lanthionine molecules are significantly more stable toward enzymatic degradation than their disulfide-bridged analogs. Bauer et al. reported in 1982 that sandostatin is many times more stable than the natural somatostatin-14 in an ultrafiltrate of rat kidney homogenate.[18] In our study, the *in vitro* stability of lanthionine-sandostatin (**1**) was compared with sandostatin against degradation by rat brain homogenate. Both compounds have clearly a prolonged duration of action. However, the lanthionine analog showed a remarkable 2.4 times longer half life than sandostatin. *In vivo* metabolism of sandostatin and lanthionine–sandostatin in rat brain tissue was studied by utilizing the cerebral microdialysis method. Both peptides yielded two extracellular metabolites detected by ESI-MS from the collected dialysates. The exocyclic residues (D-Phe and Thr-ol) show enzymatic instability; they are cleaved enzymatically from the parent compounds and provide the corresponding hexa- and heptapeptides as major metabolites. Only small portions of both drugs degraded *in vivo* in rat brain, which highlights the increased stability of these molecules.

Conclusion

Sandostatin, a highly potent, clinically used somatostatin analog, was the subject of our chemical modifications in order to develop new lead molecules with improved pharmacological profiles. The ring structure of sandostatin was reduced by elimination of one sulfur

atom from the disulfide bridge. The synthesis of these compounds was carried out on an oxime resin by using the PCOR method. The lanthionine−sandostatin, in comparison to sandostatin, showed an enhanced receptor selectivity: decreased affinity for mSSTR2b receptors and consequently 2 orders of magnitude weaker effect on the inhibition of GH release. In addition, the lanthionine−sandostatin has the same affinity for rSSTR5 as sandostatin.

The introduction of the lanthionine moiety into the sandostatin molecule increased its metabolic stability. The lanthionine−sandostatin has a longer half-life (2.4 times) in rat brain homogenates. In *in vivo* studies, two extracellular metabolites of lanthionine−sandostatin have been determined from the CNS for the first time.

In summary, the newly developed lanthionine−sandostatin octapeptides are good drug candidates on the basis of their high and selective binding affinities for somatostatin receptor SSTR5 and their increased stability against enzymatic degradation.

Experimental Section

Protected amino acids were purchased from either Bachem Bioscience or Novabiochem. All amino acids were of the L configuration if not otherwise indicated. *p*-Nitrobenzophenone oxime resin was prepared according to the literature.[45] ACS grade DCM, DMF, *i*-PrOH, EtOAc, $CHCl_3$, and MeOH were purchased from Fisher Scientific and dried over sodium aluminosilicate molecular sieves (4 Å nominal pore diameter) obtained from Sigma. DMF was treated with Amberlist-15 cation-exchange resin. TFA, TFE (Aldrich), and NMP (Applied Biosystem) were used without further purification. DIEA (Aldrich) was dried over KOH and distilled from ninhydrin. Silica gel for flash chromatography was purchased from Baker, HOBt from Merck, BOP from Novabiochem, thioanisole from Aldrich, EDC·HCl from Bachem California, and HBTU from Applied Biosystem.

Solid phase peptide syntheses were carried out batchwise either manually or by using an Applied Biosystem, Inc. Model 431A automated peptide synthesizer.

Crude and purified peptides were analyzed on precoated silica gel 6 F_{254} 0.25 mm plates (Merck) using (A) $CHCl_3$/MeOH/AcOH, 12/1/1; (B) EtOAc/BuOH/AcOH/water, 1/1/1/1; (C) *n*-BuOH/AcOH/H_2O, 4/1/1. Analytical RP-HPLC was performed on a Vydac C_{18} analytical (0.46 × 25 cm) column using 0.1% TFA in acetonitrile−water as the eluant. A linear gradient from 10 to 90% acetonitrile over 40 min, with a flow rate of 1.2 mL/min, was employed. For purification of peptides, a Vydac C_{18} semipreparative (1.0 × 25 cm) column was used with the solvent system described above.

Cbz-c[Ala$_L$Phe-D-Trp(For)Lys(Tos)Thr(Bzl)Ala$_L$]-OMe (3). BocThr(Bzl)-oxime resin (5.4 g, substitution level: 0.26 mmol/g based on picric acid titration) was deprotected with 25% TFA/DCM (v/v) and neutralized by 5% DIEA/DCM (v/v). The peptide chain was then assembled by consecutive addition of 2.5 equiv of BOP esters of BocLys(Tos)OH, Boc-D-Trp(For)OH, BocPheOH, and ZAla$_L$(TrtAla$_L$OMe)OH followed by deprotection steps. After the Trt group was removed with 25% TFA/DCM (v/v), the peptidyl resin was washed and neutralized according to the standard oxime resin protocol. The cyclization reaction was carried out by shaking the peptidyl resin in DMF−DCM (100 mL, 2/1, v/v) in the presence of 10 equiv of acetic acid at room temperature for 24 h. The cyclic peptide product was obtained from the filtrate of the reaction mixture: the solvent was removed under reduced pressure and the product was precipitated from a mixture of DMF−water. Yield: 1.07 g (65%). R_f(A): 0.73. RP-HPLC: t_R = 34.0 min (standard analytical conditions described above). FAB-MS: *m*/*z* 1173 [MH$^+$], theoretically 1173.4, ^{1}H-NMR (DMSO-d_6): δ (ppm) 0.99 (m, 2H, 2H$_\gamma$-Lys^5), 1.08 (d, 3H, *Me*-Thr^6, J = 5.9 Hz), 1.24 (m, 2H, 2H$_\delta$-Lys^5), 1.41 (m, 1H, H$_{\beta1}$-Lys^5), 1.59 (br, 1H, H$_{\beta2}$-Lys^5), 2.33 (s, 3H, *Me*-Tos), 2.57 (m, 2H, 2H$_\epsilon$-Lys^5), 2.68 (m, 1H, H$_{\beta1}$-Phe^3), 2.74 (m, 2H, 2H$_\beta$-Ala$_L^2$), 2.77 (m, 2H, H$_{\beta1}$-Ala$_L^7$ + H$_{\beta2}$-Phe^3), 2.81 (m, 1H, H$_{\beta1}$-D-Trp^4), 2.86 (m, 1H, H$_{\beta2}$-Ala$_L^7$), 2.99 (m, 1H, H$_{\beta2}$-D-Trp^4), 3.60 (s, 3H, OMe), 3.88 (quintet, 1H, H$_\beta$-Thr^6, J = 5.48 Hz), 4.48 (d, 1H, H$_{\beta1}$-CH$_2$ of BzlO-Thr^6, J = 12.3 Hz), 4.51 (d, 1H, H$_{\beta2}$-CH$_2$ of BzlO-Thr^6, J = 12.3 Hz), 4.58 (m*, 1H, H$_\alpha$-Phe^3), 4.67 (m*, 1H, H$_\alpha$-Ala$_L^2$), 5.00 (s, 2H, Cbz(C*H*$_2$)), 6.96−7.02 (m, 5H, C$_6$*H*$_5$-Phe^3), 7.21−7.28 (m, 5H, C$_6$*H*$_5$ of BzlO-Thr^6), 7.29 (d, 1H, NH-Ala$_L^2$, J = 7.8 Hz), 7.29−7.35 (m, 5H, C$_6$*H*$_5$ of Cbz), 7.35 (d, 2H of Tos ortho to SO_2), 7.36−7.41 (m, 3H, H^2 + H^5 + H^6 of Trp^4), 7.42 (t, 1H, NH$_{(\epsilon)}$-Lys^5, $J \approx$ 5 Hz), 7.53−7.58 (m, 2H, H^4 + H^7 of D-Trp^4), 7.54 (d, 1H, NH-Thr^6, $J \approx$ 8 Hz), 7.65 (d, 2H, 2H of Tos meta to SO_2), 8.17 (d, 1H, NH-Lys^5), 8.31 (d, 1H, NH-Phe^3, $J \approx$ 8 Hz), 8.33 (d, 1H, NH-Ala$_L^7$, J = 7.8 Hz), 8.41 (d, 1H, NH-D-Trp^4, $J \approx$ 8 Hz), 9.15 (s, 0.6H$^\#$, H(For)), 9.58 (s, 0.4$^\#$, H(For)). *This multiplet appears like a broad doublet of doublets. $^\#$The H of For appears splitted in two singlets, possibly because of the presence of a cis−trans isomerism involving the For group.

Cbz-D-Phe-c[Ala$_L$Phe-D-Trp(For)Lys(Tos)Thr(Bzl)Ala$_L$]-OMe (4). The protected hexapeptide (**3**, 1.0 g, 0.85 mmol) was specifically deprotected with 33% HBr/AcOH (6 mL, v/v) at 0 °C for 10 min and at room temperature for 50 min. Absolute ether (50 mL) was added to the reaction mixture, and the solution was kept at 0 °C for 1 h. The product was filtered and dried. Yield: 0.92 g (96.7%). R_f(B): 0.93. Cbz-D-Phe-OH (3.2 g, 3.29 mmol) and HOBt·H_2O (0.89 g, 6.58 mmol) were dissolved in DMF (5 mL) and cooled to 0 °C, and EDC·HCl (0.69 g, 3.62 mmol), DIEA (1.91 mL, 11.0 mmol), and the HBr salt of the above peptide (0.91 g, 0.82 mmol) were given to the reaction mixture. After being stirred for 1 h at 0 °C and overnight at room temperature, the product was precipitated by addition of water. The crude product was filtered, washed with 0.5 N HCl, 5% $NaHCO_3$, and water, and then recrystallized from DMF−ether. Yield: 0.9 g (86%). RP-HPLC: t_R = 31.8 min (standard analytical conditions described above). FAB-MS: *m*/*z* 1231 [MH$^+$], theoretically 1230.5.

Cbz-D-Phe-c[Ala$_L$Phe-D-Trp(For)Lys(Tos)Thr(Bzl)Ala$_L$]-Thr(Bzl)-ol (6). The protected heptapeptide (**4**, 0.3 g, 0.24 mmol) in DMF (6 mL) was stirred with N_2H_4·H_2O (0.6 mL) overnight under a nitrogen atmosphere. The solution was concentrated under reduced pressure, and then water was added to the reaction mixture. The precipitated hydrazide (**5**) was filtered and dried. Yield: 220 mg (60.9%). R_f(A): 0.37. RP-HPLC: t_R = 29.3 min (standard analytical conditions described above). FAB-MS: *m*/*z* 1204 [MH$^+$], theoretically 1202.5.

To a solution of the peptide hydrazide (**5**, 180 mg, 0.15 mmol) in DMF (2 mL) cooled to −15 °C were added 4 N HCl/dioxane (0.225 mL) and *tert*-butyl nitrite (0.042 mL 0.35 mmol). After the mixture was stirred for 25 min at −15 °C, HCl·H-Thr(Bzl)-ol (0.21 g, 0.9 mmol) and DIEA (0.195 mL, 1.12 mmol) were added. The reaction mixture was stirred for 1 h at −15 °C and for 48 h at 4 °C and then diluted with water. The precipitated product was filtered, washed with 1 N HCl, and 5% $NaHCO_3$, and dried. The crude material was recrystallized from DMF−ether. Yield: 185 mg (93.7%). R_f(A): 0.61. RP-HPLC: t_R = 33.1 min (standard analytical conditions described above). FAB-MS: *m*/*z* 1367 [MH$^+$], theoretically 1365.6. ^{1}H-NMR (DMSO-d_6): δ (ppm) 0.87 (q, 2H, 2H$_\gamma$-Lys^5), 0.98 (q, 3H, *Me*-Thr^6, J = 6.8 Hz), 1.04 (d, 3H, *Me*-Thr^8, J = 6.8 Hz), 1.19 (m, 2H, 2H$_\delta$-Lys^5), 1.31 (br, 1H, H$_{\beta2}$-Lys^5), 1.51 (br, 1H, H$_{\beta1}$-Lys^5), 2.36 (s, 3H, *Me*-Tos), 2.51−2.66 (m, 4H, H$_{\beta2}$-Ala$_L^2$ and H$_{\beta2}$-Ala$_L^7$ and 2H$_\epsilon$-Lys^5), 2.85−3.04 (m, 4H, H$_{\beta1}$-D-Trp^4 and H$_{\beta1}$-Ala$_L^7$ and H$_{\beta1}$-Phe^3 and H$_{\beta1}$-D-Phe^1), 3.39 (m, 1H, H$_{\beta2}$-Thr^8), 3.51 (m, 1H, H$_{\beta1}$-Thr^8), 3.74 (m, 1H, H$_\beta$-Thr^8), 3.82 (m, 1H, H$_\alpha$-Thr^8), 3.88 (m, 1H, H$_\alpha$-Lys^5), 4.03 (m, 1H, H$_\beta$-Thr^6), 4.19 (dd, 1H, H$_\alpha$-Thr^6), 4.31−4.45 (m, 2H, H$_\alpha$-D-Phe^1 and H$_\alpha$-D-Trp^4), 4.65−4.76 (m, 4H, H$_\alpha$-Ala$_L^2$ and H$_\alpha$-Phe^3 and H$_\alpha$-Ala$_L^7$ and Thr^8-ol(O*H*)), 4.90 (s, 2H, Cbz(C*H*$_2$)), 4.94 (d, 1H, Thr^6-OH), 6.90−7.34 (m, 21H, NH-Thr^6 and NH-D-Phe^1 and H^{2,5,6,7}-D-Trp(indole) and C$_6$H$_5$-Phe^3 and Cbz(C$_6$H$_5$)), 7.36 (d, 2H, H$_{ortho}$-Tos, J = 8.6 Hz), 7.39 (t, 1H, NH$_\epsilon$-Lys, J = 6.05 Hz),

7.44 (d, 1H, NH-Thr[8]-ol, J = 9.5 Hz), 7.50 (d, 1H, H[4]-D-Trp[4]-(indole), J = 8.6 Hz), 7.65 (d, 2H, H_{meta}-Tos, J = 8.6 Hz), 7.93 (d, 1H, NH-Ala_L[7], J = 8.6 Hz), 8.03 (d, 1H, NH-Phe[3], J = 6.9 Hz), 8.35 (d, 1H, NH-Ala_L[2], J = 8.6 Hz), 8.40 (d, 1H, NH-Trp[4], J = 6.91 Hz), 8.44 (d, 1H, NH-Lys[5], J = 6.9 Hz), 10.74 (s, 1H, H[1]-(indole)-D-Trp[4]).

Cbz-D-Phe-c[Ala_LPhe-D-Trp(For)Lys(Tos)Thr(Bzl)Ala_L]-Thr-NH_2 (7). The peptide hydrazide (**5**, 180 mg, 0.15 mmol) was converted to azide as described above, and then HCl·H-Thr-NH_2 (0.139 g, 0.9 mmol) and DIEA (0.195 mL, 1.12 mmol) were added to the reaction mixture. After the reaction mixture was stirred for 1 h at −15 °C and 48 h at 4 °C, the product was obtained as described in the synthesis of compound **6**. Yield: 188 mg (97.2%). R_f(A): 0.34. RP-HPLC: t_R = 32.2 min (standard analytical conditions described above). FAB-MS: m/z 1290 [MH^+] and 1312 [MNa^+], theoretically 1288.5.

H-Ala_L-Phe-D-Trp-Lys-Thr-Ala_L-OH (8). The protected hexapeptide (**3**, 117.3 mg, 0.1 mmol) was dissolved in hexamethylphosphoramide (2.5 mL) and cooled to 15 °C, and 0.5 N NaOH (1.0 mL) was added dropwise over 30 min. The reaction mixture was stirred for further 30 min at this temperature, then diluted with water (25 mL), and acidified to pH 2.5 with 2.5 N HCl at 0 °C. After being kept for 1 h at this temperature, the product was filtered, dried, and recrystallized from DMF−ether. Yield: 110 mg (97%). R_f(A) = 0.32. RP-HPLC: t_R = 13.7 min (column and eluent above; conditions: linear gradient from 50 to 75% of acetonitrile during 20 min). FAB-MS: m/z 1131 [MH^+], theoretically 1131.4.

This material was deprotected with sodium (50 mg) in liquid ammonia (75 mL). After removal of the ammonia, the residue was dissolved in water (2 mL) and the pH was adjusted to 5 with acetic acid. The solution was subjected to gel permeation chromatography (1.5 × 75 cm Sephadex G-10 eluted with 10% acetic acid) followed by RP-HPLC purification on a Vydac C-18 semipreparative column eluted with isochratic 22% acetonitrile/water containing 0.1% TFA. The pure peptide fractions were pooled and lyophilized. Yield: 38 mg (51%). RP-HPLC: t_R = 14.5 min (standard analytical conditions described above). FAB-MS: m/z 752.8 [MH^+], theoretically 753.3. NMR data are published in our accompanying paper.[44]

H-D-Phe-Ala_L-Phe-D-Trp-Lys-Thr-Ala_L-OH (9) and H-D-Phe-Ala_L-Phe-D-Trp-Lys-Thr-D-Ala_L-OH (10). The protected heptapeptide (**4**, 90.0 mg, 0.073 mmol) was dissolved in hexamethylphosphoramide (1.8 mL) and cooled to 15 °C, and 0.5 N NaOH (0.75 mL) was added dropwise over 30 min. The reaction mixture was stirred for further 30 min at this temperature, then diluted with water (20 mL), and acidified to pH 2.5 with 2.5 N HCl at 0 °C. After being kept for 1 h at this temperature, the product was filtered, dried, and recrystallized from DMF−ether. Yield: 77.2 mg (89%). R_f(A): 0.22. FAB-MS: m/z 1189 [MH^+], theoretically 1188.5.

This material was deprotected with sodium (35 mg) in liquid ammonia (50 mL). After removal of the ammonia, the rest was dissolved in water (2 mL) and the pH was adjusted to 5 with acetic acid. The solution was subjected to gel permeation chromatography (1.5 × 75 cm Sephadex G-10 eluted with 10% acetic acid) followed by RP-HPLC purification on a Vydac C-18 semipreparative column eluted with isochratic 24% acetonitrile/water containing 0.1% TFA. The two peptide fractions pooled separately and lyophilized showed identical mass spectra.

Compound 9. Yield: 17.1 mg (23.4%). R_f(C): 0.40. RP-HPLC: t_R = 16.9 min (standard analytical conditions described above). FAB-MS: m/z 900.1 [MH^+], theoretically 900.4.

Compound 10. Yield: 12.8 mg (13.7%). RP-HPLC: t_R = 16.1 min (standard analytical conditions described above). NMR data are published in our accompanying paper.[44]

H-D-Phe-Ala_L-Phe-D-Trp-Lys-Thr-Ala_L-N_2H_3 (11). The protected heptapeptide hydrazide (**5**, 70.0 mg, 0.058 mmol) was deprotected with sodium (75 mg) in liquid ammonia (50 mL) according to the protocol described for compound **8**. The crude material was purified by gel permeation chromatography (Sephadex G-10) and then by HPLC separation (on a Vydac C-18 semipreparative column eluted with isochratic 24% acetonitrile/water containing 0.1% TFA). Yield: 6.5 mg (12.2%). R_f(C) = 0.30. RP-HPLC: t_R = 14.5 min (isochratic 24% acetonitrile/water). FAB-MS: m/z 914 [MH^+] and 936 [MNa^+], theoretically 914.4.

H-D-Phe-Ala_L-Phe-D-Trp-Lys-Thr-Ala_L-Thr-ol (1). The protected octapeptide (**6**, 30.0 mg, 0.022 mmol) was deprotected with sodium (15 mg) in liquid ammonia (20 mL) according to the protocol described for compound **8**. The crude material was purified by gel permeation chromatography (Sephadex G-10) and then by HPLC separation (on a Vydac C-18 semipreparative column eluted with isochratic 24% acetonitrile/water containing 0.1 % TFA). Yield: 13.4 mg (62%). RP-HPLC: t_R = 16.4 min (standard analytical conditions described above). FAB-MS: m/z 987.1 [MH^+], theoretically 987.5. NMR data are published in our accompanying paper.[44]

H-D-Phe-Ala_L-Phe-D-Trp-Lys-Thr-Ala_L-Thr-NH_2 (2). The protected octapeptide amide (**7**, 30.0 mg, 0.023 mmol) was deprotected by sodium (15 mg) in liquid ammonia (20 mL). After removal of the ammonia the residue was desalted by gel permeation chromatography (1.5 × 75 cm Sephadex G-10 eluted with 10% acetic acid) followed by RP-HPLC purification on a Vydac C-18 semipreparative column. The pure peptide fractions were pooled and lyophilized. Yield: 15.7 mg (68%). RP-HPLC: t_R = 16.2 min (standard analytical conditions described above). FAB-MS: m/z 1000.5 [MH^+], theoretically 1000.5. NMR data are published in our accompanying paper.[44]

H-c[CysPhe-D-TrpLysThrCys]-OH (12) and H-D-Phe-c[CysPhe-D-TrpLysThrCys]OH (13). The synthesis started from Fmoc-Cys(Trt)-HMPB resin (0.75 g, 0.3 mmol/g substitution level). *Assembly protocol* was as follows: deprotection (20% piperidine/NMP, 20 + 2 × 2.5 min), wash (NMP), single coupling (4 equiv of HBTU−HOBt−DIEA, 20 min), wash (NMP). In successive couplings, FmocThr(^{t}Bu)OH, FmocLys(Boc)OH, Fmoc-D-Trp(Boc)OH, FmocPheOH, FmocCys(Trt)OH, and Boc-D-PheOH were used. *Cyclization:* Both resin-bound peptides (hexamer **14** and heptamer **15**) were treated with 10 equiv of I_2/NMP for 1 h, then washed (NMP, DCM, EtOH), and dried. *Cleavage−deprotection:* Samples (0.5 g) of both substances were subjected to final deprotection. Hexamer **14** was treated first with 20% piperidine/DMF for 25 min, and then both peptidyl resins were treated with Reagent-K (3 mL) at 0 °C for 1 h. The resins were filtered and washed with TFA. The crude products were isolated from the liquid phases by ethereal precipitation and centrifugation. The peptides were purified by RP-HPLC on a Vydac C-18 semipreparative column (eluent: acetonitrile−water mixture containing 0.1% TFA; conditions: linear gradient from 18 to 24% of acetonitrile during 20 min).

Hexapeptide 12. Yield: 16 mg (17.5%). RP-HPLC: t_R = 14.6 min (standard analytical conditions described above). FAB-MS: m/z 785.3 [MH^+], theoretically 785.3.

Heptapeptide 13. Yield: 20 mg (17.1%). RP-HPLC: t_R = 16.5 min (standard analytical conditions described above). FAB-MS: m/z 932.4 [MH^+], theoretically 932.4. NMR data are published in our accompanying paper.[44]

Mass Spectrometry. LSIMS and high-energy CID spectra were recorded on a Kratos Concept IIHH four sector tandem mass spectrometer equipped with a charge-coupled device (CCD) detector and a continuous flow probe for sample introduction. A mixture of 5% acetonitrile, 5% thioglycerol, 0.1% TFA in water was used as a liquid matrix.[46,47] For the high-energy CID experiments, He was used as the collision gas, and its pressure was adjusted to attenuate the precursor ion abundance to 30% of its initial value. The collision energy was 4 keV.

Sample Preparations. About 0.1 mg of the sample was dissolved in 50 μL of 20% acetonitrile/0.1% TFA/H_2O. A 2 μL aliquot of the sample solutions was mixed with 20 μL of liquid matrix (5% acetonitrile/5% thioglycerol/0.1% TFA in water). Then 5 μL of these solutions was injected for the LSIMS. For the high-energy CID experiments, 15 μL of the original peptide

solutions was dried, redissolved in 18 μL of 100 mM NH_4HCO_3 buffer, and incubated with chymotrypsin overnight. Two microliters of the digests was diluted with the liquid matrix and analyzed by LSIMS and high-energy CID as described above.

Enzymatic Degradation Studies. Approximately 100 nmol of peptide was added to 1 mL of rat brain homogenate (10% w/w, in artificial cerebrospinal fluid, pH 7.4), and the mixture was incubated at 37 °C in a temperature-controlled, shaking water bath. Aliquots (100 μL) were removed after 5, 15, 30, 45, 60, and 90 min of incubation, respectively, and transferred to 1.5 mL plastic centrifuge tubes containing 200 μL of ice-cold 1 M aqueous acetic acid. After centrifugation at 12500*g* for 15 min, the supernatant was removed and analyzed by microbore high-performance liquid chromatography. Analyses were done on a 1 mm × 300 mm i.d. Supelcosil LC-18 (5 μm) reversed-phase column (Supelco, Bellefonte, PA), at 216 nm. A mobile phase of water−acetonitrile containing 0.1% TFA was used in a gradient elution. HPLC calibration curves for determining concentrations were obtained by adding known amount of peptide into aliquots of brain homogenate transferred into ice-cold 1 M acetic acid solution and analyzing the supernatant after centrifugation.

***In Vivo* Metabolism of Sandostatin Analogs.** Sprague−Dawley rats (250−300 g body weight) were used to study *in vivo* metabolism in brain tissue.[43,48] A guide cannula was implanted stereotaxically under pentobarbital anesthesia into the striatum (anterior−posterior +2.4 mm and medial−lateral +3.0 mm with reference to bregma and sagittal suture, respectively, and dorsal−ventral −6.0 mm in depth from the surface of the cortex). After 5−7 days of recovery from surgery, a CMA/12 microdialysis probe fitted with a 4 mm long polycarbonate membrane (BAS, West Lafayette, IN) was inserted through the guide cannula, and a 200 μM solution of the peptide in artificial cerebrospinal fluid was perfused through the probe at a flow rate of 0.25 μL/min with a microliter syringe pump (BAS, West Lafayette, IN). The unanesthetized animal was placed in a plastic bowl, and dialysate (10−20 μL) was collected. The effluent solution containing the peptide and its *in vivo* metabolites was desalted on a reversed-phase minicolumn (1 mL Supelclean LC-18, Supelco, Bellefonte, PA) and analyzed by electrospray ionization (ESI) mass spectrometry (Vectec/PerSeptive Biosystems, Model 200 ES instrument).[49,50]

Abbreviations. The following abbreviations are used in the paper: Ala_L, one end of a lanthionine unit; Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate; ^{t}Bu, *tert* butyl; Bzl, benzyl; Cbz, benzyloxycarbonyl; COSY-DQF, double quantum-filtered correlation spectroscopy; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO-d_6, hexadeuterated dimethyl sulfoxide; EDC, 1-ethyl-3-(3′-(dimethylamino)propyl)carbodiimide; Fmoc, 9-fluorenylmethoxycarbonyl; For, formyl; HMPA, hexamethylphosphoramide; HOBt, *N*-hydroxybenzotriazole; HOHAHA, homonuclear Hartman−Hahn experiment; NMP, *N*-methylpyrrolidone; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy; TFA, trifluoroacetic acid; PCOR, peptide cyclization on an oxime resin; Tos, *p*-toluenesulfonyl; Trt, trityl or triphenylmethyl.

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Supporting Information Available: High-energy CID spectrum of the chymotrypsin-digested disulfide-bridged heptapeptide (3 pages). Ordering information is given on any current masthead page.

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