Journal of Medicinal Chemistry

Volume 42, Number 11 June 3, 1999

Articles

Highly Potent Cyclic Disulfide Antagonists of Somatostatin

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Received November 5, 1998

The search for synthetic analogues of somatostatin (SRIF) which exhibit selective affinities for the five known receptor subtypes (sst_{1-5}) has generated a large number of potent agonist analogues. Many of these agonists display good subtype selectivities and affinities for the subtypes 2, 3, and 5, with very few selective for sst_1 or sst_4. Until the recent report by Bass and co-workers (*Mol. Pharmacol.* **1996**, *50*, 709−715; erratum *Mol. Pharmacol.* **1997**, *51*, 170), no true antagonists of somatostatin had been discovered, let alone any displaying differential receptor subtype selectivity. In this present study, we further explore the effect of this putative L,^{5}D^6 antagonist motif on somatostatin octapeptide analogues with a cyclic hexapeptide core. The most potent antagonist found to date is H-Cpa-cyclo[DCys-Tyr-DTrp-Lys-Thr-Cys]-Nal-NH_2, PRL-2970 (**21**), which has an IC_{50} of 1.1 nM in a rat pituitary growth hormone in vitro antagonist assay versus SRIF (1 nM). This analogue bound to cloned human somatostatin subtype 2 receptors with a K_i of 26 nM. The highest $hsst_2$ affinity analogue was H-Cpa-cyclo-[DCys-Pal-DTrp-Lys-Tle-Cys]-Nal-NH_2, PRL-2915 (**15**), with a K_i of 12 nM (IC_{50} = 1.8 nM). This analogue was also selective for $hsst_2$ over $hsst_3$ and $hsst_5$ by factors of 8 and 40, respectively, and had no agonist activity when tested alone at concentrations up to 10 μM. Regression analysis of the binding affinities versus the observed antagonist potencies revealed high correlations for $hsst_2$ ($r = 0.65$) and $hsst_3$ ($r = 0.52$) with a less significant correlation to $hsst_5$ ($r = 0.40$). This is quite different from the somatostatin agonist analogues which show a highly significant correlation to $hsst_2$ ($r > 0.9$). Receptor-selective somatostatin antagonists should provide valuable tools for characterizing the many important physiological functions of this neuropeptide.

Introduction

The tetradecapeptide somatostatin (SRIF)[1] is a potent regulator of multiple biological functions. Although SRIF was originally isolated from mammalian hypothalamii and characterized as a potent physiological inhibitor of growth hormone (GH) secretion from the anterior pituitary, SRIF also inhibits the pancreatic secretion of glucagon and insulin[2] and the secretion of gastrin from the gut.[3] Additionally, SRIF acts as a neurotransmitter or neuromodulator in the central nervous system and peripheral tissues where it modulates neuronal firing,[4,5] release of other neurotransmitters,[6,7] motor activity,[8] and cognitive processes.[9] These biological effects of SRIF, mostly inhibitory in nature, are elicited through a series of G protein-coupled, transmembrane receptors, of which five different receptor subtypes have been characterized (sst_{1-5}).[10] These five subtypes have similar affinities for the endogenous

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10.1021/jm9806289 CCC: $18.00
Published on Web 05/06/1999

SRIF ligands but have differing distributions in various tissues.[11] The development of smaller, potent SRIF agonists led to the discovery of differing affinities of the various shortened ligands for the different receptor subtypes.[12,13] These selective agonists are currently being used to probe the physiological role of each of the receptors.[14−16] Thus far, of the five receptor subtypes for somatostatin, only sst_2 and sst_5 have been associated with specific physiological functions. SST_2 has a predominant role in mediating the release of GH as evidenced by the positive correlation between the binding affinities of a large number of small agonist analogues and their ability to inhibit GH secretion from cultured rat anterior pituitaries.[13] Inhibition of insulin secretion from rat pancreatic islets is thought to be mediated through sst_5.[17,18] However, elucidating the physiological role of each of the receptor subtypes would be greatly enhanced by the development of receptor subtype-specific antagonists.[11] Also, SRIF antagonists might provide a novel route to increasing endogenous levels of some hormones, notably growth hormone and insulin.

Some years ago, we reported the first partial antagonist of somatostatin: cyclo[Ahp-Phe-DTrp-Lys-Thr-(Bzl)].[19] This analogue was a weak antagonist at low doses, stimulating growth in female rats,[20] but was an agonist at higher doses. However, the analogue does not bind to cloned human sst receptors with any appreciable affinity.[21] However, a recent observation by Bass and co-workers has appeared to realize the goal of pure somatostatin antagonists.[22,23] They noted that a weak linear octapeptide agonist we reported recently containing 4-nitrophenylalanine (Npa), H-DPhe^5-Npa^6-Tyr^7-DTrp^8-Lys^9-Val^{10}-Phe^{11}-Thr^{12}-NH_2 (SRIF numbering),[13] had a high affinity for sst_2 but a weak effect on growth hormone levels and thus was a good candidate from which to derive an antagonist. From this compound they designed disulfide-cyclized analogues employing Npa and inverted chirality in positions 5 and 6 relative to the agonists. These compounds were shown to antagonize the action of SRIF in a cAMP accumulation assay and the somatostatin-stimulated growth of yeast cells expressing the sst_2 subtype. Of these antagonists, Ac-Npa-cyclo[DCys-Tyr-DTrp-Lys-Thr-Cys]-DTyr-NH_2 had an affinity for sst_2 comparable with that of the native hormone.[22,23] In our assay systems, the Bass analogue, H-Npa-cyclo[DCys-Tyr-DTrp-Lys-Val-Cys]-Tyr-NH_2,[22,23] gave an in vitro rat antagonist IC_{50} of 50 ± 18 nM and K_i's for $hsst_2$ and $hsst_5$ of 21 ± 5 and 98 ± 24 nM, respectively.[24] Recently, we reported on the general applicability of this putative L^5,D^6 antagonist motif on somatostatin analogues of various topologies, both linear and cyclic, but not containing Npa,[21] the presence of which was implied to be important for antagonism.[22,23] It was found that Npa is not a structural requirement for antagonism. It was also demonstrated that, indeed, the above motif is capable of converting agonists with different topologies into competitive antagonists with varying affinities and potencies.[21] The antagonists derived from cyclic agonists were found to be consistently more potent than those derived from linear sequences.[21] In this paper, we further explore the SRIF antagonist paradigm by refining this structural motif in octapeptides containing a cyclic hexapeptide core and demonstrate enhanced biological activity.

Results and Discussion

Since the discovery of the tetradecapeptide hormone somatostatin,[1] much work has been expended to ascertain the minimum structural requirements for biological activity. This has resulted in a large number of small peptide analogues, most of which are agonists.[13,25−29] Although the native tetradecapeptide hormone binds to each of the five receptor subtypes (sst_{1-5}) with approximately equal affinity, the small peptide analogues display markedly differing affinities for the five known somatostatin receptors. Almost all of these analogues contain the critical sequence DTrp^8-Lys^9, which lies in the center of a β-bend and has been shown to be crucial for receptor recognition. Most of these small analogues are cyclic, and the majority of the structure−activity relationships of these analogues can be explained in terms of changes in the intracyclic bridging region. A variety of cyclization schemes have been used to produce analogues with differing pharmacological profiles including: proline analogues and homologues,[30] β and higher homologous amino acids,[26] modifications to cystine disulfide bridging, including monosulfides[31] and trisulfides,[32] and side-chain to side-chain bridging.[33] Previously, we described a new series of linear somatostatin analogues.[13] These linear analogues have been shown to maintain the critical β-bend conformation via hydrophobic interactions between the terminal aromatic residues, by low-temperature NMR spectroscopy.[34]

Recently, an observation by Bass and co-workers[22,23] led to the first competitive antagonists of somatostatin. They described the modification of a weak agonist with high sst_2 affinity to an antagonist by the inversion of chirality at positions 5 and 6.[22,23] In a previous study,[21] we explored the effect of this putative L^5,D^6 antagonist motif on various series of somatostatin agonist analogues, both linear and cyclic. It was found that many D^5,L^6 agonists could be converted into competitive antagonists by applying this motif, the most potent of which was H-Nal-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-Nal-NH_2 (compound **1**; see Table 1). This antagonist had poor selectivity for $hsst_2$ with a binding affinity (K_i) of 81 nM and an IC_{50} of 15 nM against SRIF (1 nM) in a rat in vitro antagonist bioassay (see Table 2). In contrast, the most potent linear antagonist, H-Nal-DCpa-Tyr-DTrp-Lys-Val-Phe-Thr-NH_2, was 200-fold less potent in the in vitro antagonist assay.[21] To enhance the potency and selectivity of somatostatin antagonists and refine the structure−activity relationships, we decided to investigate further our most potent cyclic analogue, an octapeptide containing a cyclic hexapeptide core (compound **1**, see Table 1).

Position 10 in Nal^{5,12} Analogues. Previous modifications to the critical β-bend region at position 7 led to a marked increase in antagonist potency by the replacement of Tyr by Pal.[21] However, another critical residue in the β-bend region is located at position 10. In most somatostatin analogues published, this residue is usually Val or the sterically similar, but hydrophilic, Thr. Hence, we chose to investigate the effect of side-chain steric bulk on the β-bend region by modifying position 10. Replacement of Val with Gly (compound **2**)

Table 1. Analogue Amino Acid Sequences and Analytical Data

			mass spectrum M − H$^+$		HPLC[c]	
no.	code	peptide sequence	calcd[a]	obsd[b]	t_{R-1}[d]	t_{R-2}[e]
1	DC-38-48	H-Nal-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-Nal-NH$_2$[21]				
2	PRL-2876	H-Nal-cyclo[DCys-Pal-DTrp-Lys-Gly-Cys]-Nal-NH$_2$	1137.4	1136.5	30.2	26.2
3	PRL-2874	H-Nal-cyclo[DCys-Pal-DTrp-Lys-Ala-Cys]-Nal-NH$_2$	1149.9	1150.5	31.4	28.9
4	PRL-2877	H-Nal-cyclo[DCys-Pal-DTrp-Lys-Leu-Cys]-Nal-NH$_2$	1193.1	1192.6	34.8	35.8
5	PRL-2879	H-Nal-cyclo[DCys-Pal-DTrp-Lys-Nle-Cys]-Nal-NH$_2$	1193.0	1192.6	34.9	36.5
6	PRL-2875	H-Nal-cyclo[DCys-Pal-DTrp-Lys-Ile-Cys]-Nal-NH$_2$	1195.2	1192.6	33.8	36.6
7	PRL-2889	H-Nal-cyclo[DCys-Pal-DTrp-Lys-Tle-Cys]-Nal-NH$_2$	1193.2	1192.6	33.2	37.5
8	PRL-2900	H-Nal-cyclo[DCys-Bta-DTrp-Lys-Val-Cys]-Nal-NH$_2$	1232.6	1233.6	48.1	52.3
9	PRL-2891	H-Nal-cyclo[DCys-3ITyr-DTrp-Lys-Val-Cys]-Nal-NH$_2$	1319.1	1319.4	42.0	42.5
10	PRL-2908	H-Trp-cyclo[DCys-Pal-DTrp-Lys-Tle-Cys]-Nal-NH$_2$	1181.3	1181.5	30.5	34.8
11	PRL-2882	H-Fpa-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-Nal-NH$_2$	1146.9	1146.4	29.8	30.7
12	PRL-2904	H-DFpa-cyclo[DCys-Pal-DTrp-Lys-Tle-Cys]-Nal-NH$_2$	1159.1	1160.5	31.2	34.3
13	PRL-2903	H-Fpa-cyclo[DCys-Pal-DTrp-Lys-Tle-Cys]-Nal-NH$_2$[35]	1159.1	1160.5	31.1	39.0
14	PRL-2910	H-Cpa-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-Nal-NH$_2$	1162.7	1162.8	31.1	32.4
15	PRL-2915	H-Cpa-cyclo[DCys-Pal-DTrp-Lys-Tle-Cys]-Nal-NH$_2$	1176.7	1176.9	31.9	35.9
16	PRL-3020	H-Cpa-cyclo[DCys-Pal-DTrp-Lys-Thr-Cys]-Nal-NH$_2$	1164.8	1164.5	29.4	42.9
17	PRL-3052	H-Cpa-cyclo[DCys-Pal-DTrp-Lys-Tba-Cys]-Nal-NH$_2$	1190.9	1189.6	34.0	54.8
18	PRL-3023	H-Cpa-cyclo[DCys-2Pal-DTrp-Lys-Thr-Cys]-Nal-NH$_2$[f]	1164.8	1164.3	30.3	47.2
19	PRL-3024	H-Cpa-cyclo[DCys-D2Pal-DTrp-Lys-Thr-Cys]-Nal-NH$_2$[f]	1164.8	1165.7	31.4	51.2
20	PRL-2972	H-Cpa-cyclo[DCys-Phe-DTrp-Lys-Thr-Cys]-Nal-NH$_2$	1163.8	1163.9	38.6	58.1
21	PRL-2970	H-Cpa-cyclo[DCys-Tyr-DTrp-Lys-Thr-Cys]-Nal-NH$_2$	1179.8	1180.5	33.8	48.4
22	PRL-2859	H-Fpa-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-Fpa-NH$_2$	1114.3	1114.3	26.0	24.5
23	PRL-2872	H-Fpa-cyclo[DCys-His-DTrp-Lys-Val-Cys]-Fpa-NH$_2$	1104.7	1103.3	25.3	14.3
24	PRL-2888	H-DFpa-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-DFpa-NH$_2$	1116.8	1114.3	25.6	26.2
25	PRL-2858	H-3Fpa-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-3Fpa-NH$_2$	1114.3	1114.3	25.9	24.4
26	PRL-2869	H-3Fpa-cyclo[DCys-His-DTrp-Lys-Val-Cys]-3Fpa-NH$_2$	1104.2	1103.3	25.1	13.9
27	PRL-2857	H-2Fpa-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-2Fpa-NH$_2$	1114.9	1114.3	24.4	22.0
28	PRL-2868	H-2Fpa-cyclo[DCys-His-DTrp-Lys-Val-Cys]-2Fpa-NH$_2$	1103.7	1103.3	23.6	11.9
29	PRL-2894	H-Cpa-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-Cpa-NH$_2$	1147.2	1147.1	29.7	28.5
30	PRL-2917	H-Cpa-cyclo[DCys-Pal-DTrp-Lys-Tle-Cys]-Cpa-NH$_2$	1161.1	1161.2	30.8	34.4
31	PRL-2918	H-DCpa-cyclo[DCys-Pal-DTrp-Lys-Tle-Cys]-DCpa-NH$_2$	1163.5	1161.2	30.7	35.9
32	PRL-2905	H-Bpa-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-Bpa-NH$_2$	1236.0	1236.1	30.6	31.7
33	PRL-2907	H-Iph-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-Iph-NH$_2$	1333.2	1330.1	32.2	34.1
34	PRL-2856	H-Pfp-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-Pfp-NH$_2$	1259.0	1258.1	31.7	32.8
35	PRL-2862	H-Pfp-cyclo[DCys-His-DTrp-Lys-Val-Cys]-Pfp-NH$_2$	1248.7	1247.1	31.0	23.6
36	PRL-3064	H-Pfp-cyclo[DCys-2Pal-DTrp-Lys-Val-Cys]-Pfp-NH$_2$[f]	1257.9	1258.1	31.4	52.2
37	PRL-2855	H-Pfp-cyclo[DCys-D2Pal-DTrp-Lys-Val-Cys]-Pfp-NH$_2$[f]	1257.9	1258.1	33.8	35.6
38	PRL-2870	H-Bip-cyclo[DCys-Tyr-DTrp-Lys-Ile-Cys]-Bip-NH$_2$	1261.8	1259.6	44.2	45.7
39	PRL-2902	H-Ypa-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-Ypa-NH$_2$	1128.3	1128.3	22.6	19.6
40	PRL-2896	H-Igl-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-Igl-NH$_2$	1133.0	1130.5	28.8	30.3
41	PRL-2878	H-Tic-cyclo[DCys-Tyr-DTrp-Lys-Val-Cys]-Tic-NH$_2$	1117.3	1117.4	31.8	26.5
42	PRL-2897	H-Nal-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-DDip-NH$_2$	1204.1	1204.5	35.5	37.6
43	PRL-2898	H-Nal-cyclo[DCys-Tyr-DTrp-Lys-Val-Cys]-DDip-NH$_2$	1219.7	1219.5	41.2	41.5
44	PRL-2883	H-Nal-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-Fpa-NH$_2$	1149.4	1146.4	28.7	28.1

[a] Theoretical molecular weight (M − H$^+$, Da). [b] Observed molecular weight (M − H$^+$, Da). [c] Reversed-phase HPLC (C-18, 5 μm, 4.6 × 250 mm, λ = 215 nm) retention times (min). Each compound was found to have a purity of >97% in both HPLC systems. [d] HPLC-1 elution system: A, 0.1% TFA; B, 0.1% TFA in 80% MeCN; 10% B to 70% B at 1% min^{-1} and 1.5 mL min^{-1}. [e] HPLC-2 elution system: C, 5% MeCN in TEAP (0.1 M, pH 3); D, 20% C in MeCN, 20% D to 80% D at 1% min^{-1} and 1.5 mL min^{-1}. [f] Tentative assignment of 2Pal chirality.

was accompanied by a large (100-fold) reduction in in vitro potency to 1.5 μM, though with only a halving of affinity for hsst$_2$ (see Table 2). Ala^{10} (compound **3**) caused a smaller loss in antagonist activity than Gly (IC$_{50}$ = 425 nM), with a 2.5-fold reduction in affinity for hsst$_2$, and markedly less affinity for hsst$_5$ (**3**, K_i = 1860 nM). The substitution of Leu in position 10 (**4**) was accompanied by a decrease in antagonist activity (IC$_{50}$ = 48 nM) and a marked reduction in affinity for hsst$_2$ relative to compound **1**. Nle^{10} (**5**) resulted in a similar IC$_{50}$ to that of the Leu^{10} analogue but restored the affinity for hsst$_2$ and hsst$_3$. Replacement of Val^{10} with Ile (**6**) gave an antagonist with potency and affinity for hsst$_3$ similar to that of the parent compound (**1**) although it abolished affinity for hsst$_2$. The unusual amino acid *tert*-leucine (Tle^{10}) caused the antagonist potency to double relative to Val^{10} (**7**, IC$_{50}$ = 8.4 nM), and the affinity for hsst$_2$ and hsst$_4$ was increased. In an extension of the previous studies involving aromatic modifications to position 7,[21] the replacement of Pal in analogue **1** with the sulfur analogue of tryptophan (Bta) was accompanied by marked reductions in IC$_{50}$ and K_i for hsst$_2$, hsst$_3$, and hsst$_5$ (compound **8**). The insertion of a nonradioactive version of the moiety frequently generated in radiolabeling peptides, 3-I-Tyr^7 (**9**), in place of Pal^7 in compound **1** retained the antagonist activity, but the binding affinities for types 2 and 3 were approximately halved.

Aromatic^5,Nal^{12} Analogues. In the agonist series of analogues with similar topology, the presence of large hydrophobic aromatic amino acids in position 5 is important for enhanced biological activity.[13] Additionally, many analogues also contain a hydrophobic aromatic amino acid at position 12. In a previous paper,[21] we reported that although both the agonists and antagonists share a propensity for terminal hydrophobic amino acids, the preferred chirality differs. In general, agonists of this topological class prefer a D amino acid in position 5, whereas the antagonists favor L amino acids in the same location. To study the effect of steric

Table 2. Binding Affinities (K_i) for the Cloned Human sst_{1-5} Receptors and Rat Antagonist IC_{50} Data versus Substitution at Positions 5, 7, 10, and 12 in H-W[5]-cyclo[D-Cys-X[7]-D-Trp-Lys-Y[10]-Cys]-Z[12]-NH_2

	substitution				K_i[a] ± SEM (nM)					antagonist IC_{50} ± SEM (*n*)[b]
no.	5	7	10	12	$hsst_1$	$hsst_2$	$hsst_3$	$hsst_4$	$hsst_5$	(nM)
1	Nal	Pal	Val	Nal	348 ± 158	81 ± 12	171 ± 46	>1000	524 ± 175	15 ± 3.6 (5)
2	Nal	Pal	Gly	Nal	413 ± 73	163 ± 11	192 ± 24	1570	382 ± 214	1510 ± 425 (2)
3	Nal	Pal	Ala	Nal	1200 ± 196	203 ± 30	379 ± 30	>1000	1860	425 ± 25 (2)
4	Nal	Pal	Leu	Nal	415 ± 76	543 ± 11	243 ± 54	728	968 ± 166	48 ± 21 (5)
5	Nal	Pal	Nle	Nal	846 ± 639	76 ± 16	126 ± 13	672	814	38 ± 11 (3)
6	Nal	Pal	Ile	Nal	172 ± 54	>1000	152 ± 71	1040	847 ± 94	12 ± 5.8 (3)
7	Nal	Pal	Tle	Nal	>1000	35 ± 5.5	371 ± 125	145	631 ± 98	8.4 ± 0.9 (4)
8	Nal	Bta	Val	Nal	>1000	418 ± 112	709 ± 328	>1000	1400	403 ± 144 (3)
9	Nal	3ITyr	Val	Nal	>1000	194 ± 0.5	482 ± 142	>1000	468 ± 117	20 (1)
10	Trp	Pal	Tle	Nal	>1000	84 ± 6.2	210 ± 42	>1000	2250	32 ± 3.0 (2)
11	Fpa	Pal	Val	Nal	484	35 ± 8.1	271 ± 185	>1000	287	6.5 ± 1.8 (4)
12	DFpa	Pal	Tle	Nal	>1000	132 ± 18	555 ± 16	888	260	20 ± 7.5 (2)
13	Fpa	Pal	Tle	Nal	>1000	26 ± 3.1	231 ± 102	>1000	535 ± 116	2.5 ± 0.6 (11)[c]
14	Cpa	Pal	Val	Nal	>1000	25 ± 9.7	85 ± 1.2	1590	240	2.6 ± 0.9 (4)
15	Cpa	Pal	Tle	Nal	>1000	12 ± 1.3	100 ± 57	895	520	1.8 ± 0.5 (5)[c]
16	Cpa	Pal	Thr	Nal	1400	12 ± 2.2	38 ± 2.4	>1000	140 ± 4.6	2.6 ± 0.7 (3)
17	Cpa	Pal	Tba	Nal	238	166 ± 66	175	nd[d]	110 ± 34	125 ± 15 (2)
18	Cpa	2Pal[e]	Thr	Nal	1030	14 ± 1.1	53 ± 1.3	>1000	70 ± 12	2.7 ± 1.0 (3)
19	Cpa	D2Pal[e]	Thr	Nal	1690	233 ± 2.0	108 ± 60	nd	204 ± 49	135 ± 41 (2)
20	Cpa	Phe	Thr	Nal	443 ± 202	51 ± 18	290 ± 56	>1000	87 ± 50	1.4 ± 0.2 (7)
21	Cpa	Tyr	Thr	Nal	>1000	26 ± 6.8	93 ± 4.8	>1000	48 ± 11	1.1 ± 0.2 (7)
22	Fpa	Pal	Val	Fpa	435 ± 322	20 ± 2.7	120 ± 2.5	>1000	369	87 ± 36 (5)
23	Fpa	His	Val	Fpa	1950 ± 1190	31 ± 9.5	269 ± 87	847	173	105 ± 5 (2)
24	DFpa	Pal	Val	DFpa	>1000	106 ± 9.6	838 ± 665	>1000	694	74 ± 19 (3)
25	3Fpa	Pal	Val	3Fpa	82 ± 13	117 ± 48	495 ± 174	>1000	748	77 ± 28 (3)
26	3Fpa	His	Val	3Fpa	60 ± 16	103 ± 25	397 ± 193	>1000	734	137 ± 22 (3)
27	2Fpa	Pal	Val	2Fpa	186 ± 45	241 ± 11	385 ± 72	>1000	1580	114 ± 17 (3)
28	2Fpa	His	Val	2Fpa	37 ± 9.5	176 ± 88	382 ± 181	>1000	972 ± 411	393 ± 92 (3)
29	Cpa	Pal	Val	Cpa	>1000	32 ± 4.3	51 ± 42	301	139 ± 50	3.0 ± 0.8 (6)
30	Cpa	Pal	Tle	Cpa	>1000	19 ± 2.6	58 ± 13	1640	140	5.9 ± 1.6 (3)
31	DCpa	Pal	Tle	DCpa	>1000	56 ± 6.0	1190 ± 321	2880	153	71 ± 13 (3)
32	Bpa	Pal	Val	Bpa	374 ± 114	16 ± 4.8	57 ± 19	3310	66	2.7 ± 0.4 (4)
33	Iph	Pal	Val	Iph	575 ± 155	32 ± 3.7	18 ± 4.3	436	48 ± 25	4.0 ± 1.0 (6)
34	Pfp	Pal	Val	Pfp	525 ± 199	633 ± 148	111 ± 20	176	960	292 ± 135 (5)
35	Pfp	His	Val	Pfp	69 ± 12	637 ± 71	124 ± 48	>1000	926 ± 228	2410 ± 880 (3)[f]
36	Pfp	2Pal[d]	Val	Pfp	568 ± 422	>1000	170 ± 25	nd	918 ± 342	765 ± 82 (2)
37	Pfp	D2Pal[d]	Val	Pfp	34 ± 11	692 ± 78	93 ± 32	nd	1380 ± 383	na[g]
38	Bip	Tyr	Ile	Bip	>1000	2230 ± 1230	1350 ± 176	>1000	689	2110 ± 1150 (3)
39	Ypa	Pal	Val	Ypa	>1000	99 ± 6.4	103 ± 77	>1000	304	51 ± 25 (3)
40	Igl	Pal	Val	Igl	736 ± 265	252 ± 31	568 ± 108	682 ± 318	469 ± 421	81 ± 60 (2)
41	Tic	Tyr	Val	Tic	1030 ± 26	345 ± 73	802 ± 120	>1000	>1000	na[h]
42	Nal	Pal	Val	DDip	>1000	37 ± 4.7	1600 ± 599	>1000	725	91 ± 29 (3)
43	Nal	Tyr	Val	DDip	>1000	16 ± 0.6	1560 ± 557	>1000	461	65 ± 23 (3)
44	Nal	Pal	Val	Fpa	1060	69 ± 6.2	166 ± 41	>1000	376	150 ± 50 (2)

[a] Expressed as the mean ± SEM; single values indicate the results of one binding experiment. [b] Rat in vitro antagonist IC_{50} (nM) versus SRIF (1.0 nM), expressed as the mean ± SEM of *n* separate dose−response curves. [c] No agonist activity when tested alone at concentrations up to 10 μM. [d] Not determined. [e] Tentative assignment of 2Pal chirality. [f] Not a full antagonist at doses up to 10 μM. [g] Not an antagonist at doses up to 10 μM. [h] Not an antagonist at doses up to 100 μM; found to be an agonist with IC_{50} = 403 nM.

bulk at the N-terminal exocyclic position, we made a series of Nal[12] analogues containing a variety of aromatic amino acids in position 5.

The substitution of Trp for Nal[5] with Tle[10] (**10**) caused a 4-fold loss of antagonist activity and affinity for $hsst_5$ with a smaller reduction in affinity for $hsst_2$ relative to parent compound **7**. The placement of Fpa[5] (**11**) in compound **1** instead of Nal[5] caused a halving in IC_{50} to 6.5 nM, and the affinity for $hsst_2$ was increased to 35 nM. Inversion of the chirality of Fpa[5] (**12**) was accompanied by a 3-fold loss of antagonistic activity as expected when compared with the L analogue **12** (IC_{50} = 20 nM). The additional replacement of Val[10] by Tle[10], together with Fpa[5] (**13**), was accompanied by a decrease in IC_{50} to 2.5 nM and a slight increase in $hsst_2$ affinity relative to the parent analogue **7**. This compound (**13**) was the first antagonist to show some selectivity for $hsst_2$, with $hsst_2/hsst_3$ and $hsst_2/hsst_5$ K_i ratios of 8 and 20, respectively.[35] The introduction of Cpa[5] in place of Nal[5] (**14**) resulted in a potent analogue with an IC_{50} of 2.6 nM with increased affinity for $hsst_2$, $hsst_3$, and $hsst_5$ relative to the parent analogue **1**. As in the case of the Nal[12] analogues (**1** and **7**), the incorporation of Tle[10] with Cpa[5] (**15**) led to an improvement in $hsst_2$ affinity, but the IC_{50} was not improved significantly. Cpa[5],Thr[10] (**16**) was equipotent with Cpa[5],Val[10] (**14**) in the antagonist assay, although its $hsst_2$ binding affinity was improved by a factor of 2. Substitution of the bulky *tert*-butylalanine in position 10 (**17**) led to a marked reduction in antagonist activity and affinity for $hsst_2$, although the affinities for $hsst_1$ and $hsst_5$ were improved relative to Val[10] (**14**).

3-Pyridylalanine in position 7 was shown in the previous paper to be superior to Tyr[7] in terms of the antagonistic potency.[21] Interestingly, during the synthesis of an *o*-Pal analogue Cpa[5],2Pal[7],Thr[10] (**18**), the incorporation of Boc-2Pal was accompanied by an

intense ninhydrin-like blue coloration during the coupling. The coloration washed out following the completion of the reaction, and the synthesis completed as usual. HPLC chromatography of the cleaved, crude peptide showed the presence of two closely eluting peaks with identical peak areas and molecular weights, suggesting racemization had occurred during the coupling of 2Pal. However, these diastereoisomers were separated readily during the standard chromatographic purification step. The optical configuration of each diastereoisomer was tentatively inferred from a comparison of the HPLC elution behaviors with two model pairs of somatostatin analogues, synthesized separately as diastereoisomers of known optical configuration,[36] and of the observed biological activities. In each model case, the L configuration diastereoisomer eluted before the D form from a TFA/C-18 HPLC system. The faster eluting analogue, tentatively identified as the L2Pal^7 diastereoisomer (**18**), was equipotent with Pal^7 (**16**), and the affinity for hsst$_2$ was not significantly different. The slower eluting analogue, tentatively identified as the D2Pal^7 diastereoisomer (**19**), demonstrated a major loss of antagonism (IC_{50} = 135 nM) with reductions in the affinities for all the receptor subtypes. Replacement of Pal in position 7 with Phe (**20**) in the Cpa^5,Thr^{10} parent analogue (**16**) was accompanied by a halving of the IC_{50} to 1.4 nM and a 4-fold drop in affinity for hsst$_2$ to 51 nM. The substitution of Tyr^7 (**21**) produced a highly active antagonist with an IC_{50} of 1.1 nM and increased affinity for hsst$_5$, though with one-half the affinity for hsst$_2$ as Pal^7 (**16**).

Aromatic^{5,12} Analogues. To probe the effect of steric bulk at both exocyclic positions, we made a series of analogues, doubly substituted with various aromatic amino acids of differing sizes. The substitution of fluorophenylalanine (Fpa) in both exocyclic positions 5 and 12 with Pal^7 (**22**) produced a moderately active antagonist with an IC_{50} of 87 nM. Replacement of Pal by His (**23**) had little effect on the antagonism and affinity for hsst$_2$, while markedly reducing the affinity for hsst$_1$. Inversion of the chirality of both Fpa moieties together with Pal^7 (**24**), also had little effect on antagonism but noticeably reduced affinities for hsst$_2$, hsst$_3$, and hsst$_5$. Incorporation of the *m*-Fpa isomer, 3Fpa, in the terminal positions 5 and 12 (**25**) also produced an analogue with a similar antagonist activity to Fpa^{5,12} (**22**), but inferior hsst$_2$, hsst$_3$, and hsst$_5$ affinities. However, the affinity for hsst$_1$ was much improved to 82 nM. The additional incorporation of His^7 with 3Fpa^{5,12} (**26**) again reduced antagonist activity (IC_{50} = 137 nM) and increased hsst$_1$ affinity. In the case of the *o*-Fpa isomer (2Fpa) in positions 5 and 12 (**27**), the antagonism was decreased relative to the *para* analogue (**22**). The additional substitution of His^7 (**28**) increased the affinity for hsst$_1$, although the antagonist activity was reduced as expected (IC_{50} = 393 nM).

Substitution of chlorophenylalanine in both exocyclic positions (**29**) produced a 5-fold increase in antagonist activity (IC_{50} = 3 nM) relative to the Nal^{5,12} parent compound (**1**). Replacement of Val^{10} by Tle^{10} (**30**) halved the antagonism and doubled the affinity for hsst$_2$. The inversion of the chirality of the Cpa^{5,12} moieties, together with Tle^{10} (**31**), reduced the antagonism by more than 10-fold relative to the L form (**30**) and markedly reduced the affinity for hsst$_3$. Bromophenylalanine substituted in both terminal positions (**32**) gave an analogue with a similar IC_{50}, but with increased affinity for hsst$_2$ and hsst$_5$ relative to the Cpa analogue (**29**). Iodophenylalanine (**33**) also gave a similar IC_{50} to the Cpa analogue (**29**). However, this analogue had the highest affinities for hsst$_3$ and hsst$_5$ of the halogenated phenylalanine analogues.

Incorporation of the hydrophobic amino acid pentafluorophenylalanine (Pfp) in positions 5 and 12 (**34**) produced a poor antagonist (IC_{50} = 292 nM) compared with the Nal parent compound (**1**). The analogue displayed weak affinity for receptor subtypes 3 and 4, with little affinity for the other subtypes. Replacement of Pal^7 by His (**35**) further reduced the antagonism such that the analogue could not fully block the action of SRIF at the doses tested (up to 10 μM). However, affinity for hsst$_1$ was markedly improved. Incorporation of Boc-2Pal in this Pfp^{5,12} analogue was again accompanied by racemization, leading to a pair of diastereoisomers, which were readily separated by HPLC. Chirality was tentatively assigned as before. The faster eluting analogue, tentatively assigned as L2Pal^7 (**36**), gave a weak antagonist analogue which bound weakly to hsst$_3$ but did not bind to hsst$_2$. The slower eluting analogue, tentatively assigned as D2Pal^7 (**37**), produced a compound which, as expected, was not an antagonist at doses up to 10 μM. The bulky biphenylalanine (Bip) in both terminal positions with Ile^{10} (**38**) produced a very weak antagonist (IC_{50} = 2.1 μM) with little affinity for any of the receptor subtypes. Ypa^{5,12} (**39**) gave a modest antagonist with an IC_{50} of 51 nM. Substitution of Igl in both positions (**40**) produced a moderate antagonist with an IC_{50} of 81 nM but with weak affinity for all receptor types. Unexpectedly, the constrained Trp analogue, Tic^{5,12},Tyr^7 (**41**), was a feeble agonist (IC_{50} = 403 nM) with weak affinity for hsst$_2$. This compound exhibited no antagonism at the highest dose tested (100 μM).

In the previous paper, we investigated the effect of the branched amino acid diphenylalanine in position 5.[21] Substitution of DDip at position 12 with Nal^5 gave an antagonist with an IC_{50} of 91 nM and an affinity for hsst$_2$ of 37 nM (**42**), slightly more active than the His^7 analogue reported in the previous paper.[21] The affinity for sst$_2$ doubled, but the antagonism was unchanged on the additional substitution of Tyr^7 (**43**). The incorporation of Fpa^{12} with Nal^5 (**44**) reduced the antagonism by 20-fold from the Fpa^5,Nal^{12} analogue (**11**) demonstrating a distinct preference for a larger, unbranched hydrophobic moiety at the C-terminus.

Correlation of Binding Affinities with Agonist and Antagonist Activity. Some years ago, we reported a highly significant correlation (r = 0.93) between the binding affinity for cloned mouse sst$_2$ receptors and the growth hormone release-inhibiting potency of the somatostatin agonists in a rat in vitro pituitary cell assay.[13] These agonists have since been reassayed against both the cloned rat and cloned human sst$_{1-5}$ receptors, and the correlation results versus the rat in vitro growth hormone release-inhibiting potencies are given in Table 3.[37] All ill-defined K_i values (e.g. >1000) were dropped from the correlation analyses. With the cloned human receptor affinity data, a strong correlation was found between the rat in vitro growth hormone

Table 3. Correlations between Binding Affinities (K_i) for Cloned Rat and Human Somatostatin Receptors sst_{1-5} and Rat Agonist IC_{50} Potencies for Agonists and between Cloned Human Somatostatin Receptors sst_{1-5} and Rat Antagonist IC_{50} Potencies for Antagonists

receptor species	correlation coefficient (r)				
	sst_1	sst_2	sst_3	sst_4	sst_5
		Agonists[a]			
rat	nd[c]	0.94	0.89	nd	0.57
human	0.02	0.91	0.14	0.11	0.35
		Antagonists[b]			
human	0.02	0.65	0.52	0.07	0.40

[a] 34 agonists taken from ref 13 with recent rat and human receptor subtype binding data from ref 49. [b] Pooled antagonist binding data from this paper (41 unique antagonists) and the prior paper (26 antagonists) in ref 21 (Hocart et al. *J. Med. Chem.* **1998**, *41*, 1146−1154). [c] Not determined.

release-inhibiting potency of the agonists and $hsst_2$ ($r = 0.91$) with a less significant correlation to $hsst_5$ ($r = 0.35$). No significant correlations were found with $hsst_1$, $hsst_3$, or $hsst_4$ (r's < 0.16). For the agonist cloned rat receptor binding affinity data, again the highest correlation was obtained with subtype 2 ($r = 0.94$), but significant correlations were also found with $rsst_3$ and $rsst_5$ ($r = 0.89$ and 0.57, respectively).

In the case of somatostatin antagonists, these current analogues (41 compounds) and the antagonist analogues from our prior publication[21] (26 antagonists) show a different pattern of correlations between the rat in vitro antagonist potencies and the binding affinities for the cloned human receptor subtypes. These data are summarized in Table 3. The antagonists show a reduced preference for $hsst_2$ and major correlations for $hsst_3$ and $hsst_5$ relative to the agonists. This correlation pattern with the cloned human receptors is more like that found with rat receptors in the agonist series.

The study of SRIF agonists during the last 2 decades has produced a large number of agonist analogues of various ring sizes and some linear compounds.[13,25−28] More recently, SRIF agonists and antagonists have both been developed using cloned human receptor binding affinities to guide the synthesis of more active and selective compounds.[13,21] This has culminated in the synthesis of receptor subtype-selective agonist analogues.[12,13] The few potent antagonists thus far discovered have not yet produced truly subtype-specific analogues, although as can be seen from this work, $hsst_2$ partially selective analogues are beginning to be described. Thus it is not so surprising to find the lack of a dominant correlation between the observed rat in vitro antagonist IC_{50} values and the measured K_i affinities for any one of the cloned human receptor subtypes. It is hoped that routine functional assays for all five SRIF subtypes will be developed and thus provide an invaluable aid in the future refinement of more specific analogues.

Conclusion

The substitution of the same hydrophobic aromatic amino acid at both exocyclic positions shows a preference for the heavier, halogen-substituted phenylalanines (analogues ranked by increasing IC_{50} are shown in Table 4). Among the *p*-halogen-substituted phenylalanines, the fluoro compound (**22**) was much less active in the antagonist assay than the heavier halogen-substituted analogues. The chloro, bromo, and iodo analogues (**29**, **32**, and **33**, respectively) gave similar IC_{50} values and were 20-fold more potent than the fluoro analogue (**22**). Interestingly, the $hsst_2$ binding affinities for the *para*-halogenated compounds were quite similar. *o*-, *m*-, and *p*-fluoro substitution (**27**, **25**, and **22**, respectively) had little effect on the antagonist IC_{50}, but the *para* compound (**22**) had a much greater affinity for $hsst_2$. Pentafluoro substitution was accompanied by a marked reduction in $hsst_2$ affinity and antagonism. The β-branched analogue diphenylalanine (Dip) was the least active analogue of the group with little binding affinity for any receptor subtype.

A rank ordering of the most potent analogues in the rat in vitro antagonist assay ($IC_{50} < 5$ nM, see Table 5) is dominated by peptides containing a large hydrophobic amino acid, most often Nal, at the C-terminus, with a halogenated phenylalanine analogue at the N-terminus. The most active compounds ($IC_{50} < 2$ nM) contain Nal[12] exclusively, with the smaller Cpa at position 5. This is in contrast to agonist octapeptide analogues, which frequently contain smaller, or hydrophilic, amino acids at the C-terminus.[13] Although compounds **20** and **21** were found to be the most potent in the rat antagonist bioassay, they are inferior to compounds **15** and **13** in terms of their receptor subtype selectivities. These latter analogues (**15** and **13**) display selectivities for $hsst_2$ over $hsst_3$ by a factor of 8 and for $hsst_2$ over $hsst_5$ by factors of 40 and 20, respectively. The substitutions at positions 7 and 10 are responsible for the differences in selectivity between the most selective compound (**15**) and the most potent antagonists (**20** and **21**). Analogues **13** and **15** have also been found to be devoid of any agonist activity when tested alone at concentrations up to 10 μM. Subtype-selective agonists have now been described for all receptor subtypes except type 4.[38,39] These are the first potent antagonists to display partial subtype selectivity. We are continuing to investigate the structure−activity relationships of the somatostatin antagonists to further refine the subtype selectivities and antagonistic potencies to help explain these receptor correlation observations. As more selective analogues are discovered and additional functional subtype-selective assays developed, the role of each of the receptor subtypes may be further probed and potential pharmaceutical agents developed.

Experimental Section

Abbreviations. The nomenclature for the somatostatin receptor subtypes is in accordance with the recommendations of IUPHAR,[40] in which sst_4 refers to the receptor originally cloned by Bruno et al.[41] and sst_5 refers to the receptor cloned by O'Carroll et al.[17,42] Abbreviations of the common amino acids are in accordance with the recommendations of IUPAC−IUB.[43] Additional abbreviations: Bip, biphenylalanine; Bpa, 4-bromophenylalanine; Bta, benzothienylalanine; Cpa, 4-chlorophenylalanine; Dip, 3,3-diphenylalanine; Fpa, 4-fluorophenylalanine; 3Fpa, 3-fluorophenylalanine; 2Fpa, 2-fluorophenylalanine; Igl, 2-indanylglycine; Iph, 4-iodophenylalanine; 3-I-Tyr, 3-iodotyrosine; Nal, 3-(2-naphthyl)alanine; Npa, 4-nitrophenylalanine; 2Pal, 2-pyridylalanine; Pal, 3-pyridylalanine; Pfp, pentafluorophenylalanine; Tba, *tert*-butylalanine; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Tle, *tert*-leucine; Ypa, 4-cyanophenylalanine.

Materials. 4-Methylbenzhydrylamine hydrochloride resin (0.41 or 0.29 mequiv g^{-1}) was obtained from Advanced ChemTech Inc., Louisville, KY. N^{α}-*tert*-Butyloxycarbonyl (Boc)-

Table 4. Effect of the Double Substitution of an Amino Acid at Positions 5 and 12 in H-X[5]-cyclo[DCys-Pal-DTrp-Lys-Val-Cys]-X[12]-NH_2

		$K_i \pm$ SEM (nM)					antagonist $IC_{50} \pm$ SEM (n)[b]
no.[a]	X	$hsst_1$	$hsst_2$	$hsst_3$	$hsst_4$	$hsst_5$	(nM)
32	Bpa	374 ± 114	16 ± 4.8	57 ± 19	3310	65	2.7 ± 0.4 (4)
29	Cpa	>1000	32 ± 4.3	50 ± 42	301	139 ± 50	3.0 ± 0.8 (6)
33	Iph	575 ± 155	32 ± 3.6	18 ± 4.3	436	48 ± 25	4.0 ± 1.0 (6)
1	Nal	348 ± 158	81 ± 12	171 ± 45	>1000	524 ± 175	15 ± 3.6 (5)
39	Ypa	>1000	99 ± 6.4	103 ± 77	>1000	304	51 ± 25 (3)
25	3Fpa	82 ± 13	117 ± 48	495 ± 174	>1000	748	77 ± 28 (3)
40	Igl	736 ± 265	252 ± 31	568 ± 108	682 ± 318	469 ± 421	81 ± 60 (2)
22	Fpa	435 ± 322	20 ± 2.7	120 ± 2.5	>1000	369	87 ± 36 (5)
27	2Fpa	186 ± 45	241 ± 11	385 ± 72	>1000	1580	114 ± 17 (3)
34	Pfp	525 ± 199	633 ± 148	111 ± 20	176	960	292 ± 135 (5)
36[c]	Dip	>1000	1410 ± 406	1300 ± 294	>1000	2030	500 ± 120 (2)

[a] Compounds are ranked by mean IC_{50} values. [b] Antagonist IC_{50} (nM) versus SRIF (1.0 nM). [c] Compound **36** (RJ-01-76) from the previous antagonist paper: Hocart et al. *J. Med. Chem.* **1998**, *41*, 1146−1154 (ref 21).

Table 5. Potent Analogues with Rat Antagonist IC_{50} < 5 nM.

	substitution				K_i[b] ± SEM (nM)					antagonist $IC_{50} \pm$ SEM (n)[c]
no.[a]	5	7	10	12	$hsst_1$	$hsst_2$	$hsst_3$	$hsst_4$	$hsst_5$	(nM)
21	Cpa	Tyr	Thr	Nal	>1000	26 ± 6.8	93 ± 4.8	>1000	48 ± 11	1.1 ± 0.2 (7)
20	Cpa	Phe	Thr	Nal	443 ± 202	51 ± 18	290 ± 56	>1000	87 ± 50	1.4 ± 0.2 (7)
15	Cpa	Pal	Tle	Nal	>1000	12 ± 1.3	100 ± 57	895	520	1.8 ± 0.5 (5)
13	Fpa	Pal	Tle	Nal	>1000	26 ± 3.1	231 ± 102	>1000	535 ± 116	2.5 ± 0.6 (11)
14	Cpa	Pal	Val	Nal	>1000	25 ± 9.7	85 ± 1.2	1590	240	2.6 ± 0.9 (4)
16	Cpa	Pal	Thr	Nal	1400	12 ± 2.2	38 ± 2.4	>1000	139 ± 4.6	2.6 ± 0.7 (3)
32	Bpa	Pal	Val	Bpa	374 ± 114	16 ± 4.8	57 ± 19	3310	66	2.6 ± 0.4 (4)
18	Cpa	2Pal[d]	Thr	Nal	1030	14 ± 1.1	53 ± 1.3	>1000	70 ± 12	2.7 ± 1.0 (3)
29	Cpa	Pal	Val	Cpa	>1000	32 ± 4.3	51 ± 42	301	139 ± 50	3.0 ± 0.8 (6)
33	Iph	Pal	Val	Iph	575 ± 155	32 ± 3.7	18 ± 4.3	436	48 ± 25	4.0 ± 1.0 (6)

[a] All compounds are ranked by mean IC_{50} values. [b] Expressed as the mean ± SEM; single values indicate the results of one binding experiment. [c] Rat in vitro antagonist IC_{50} (nM) versus SRIF (1.0 nM), expressed as the mean ± SEM of n separate dose−response curves. [d] Tentative assignment of 2Pal chirality.

protected amino acids were purchased from Bachem Inc., Torrance, CA, Advanced ChemTech Inc., and Synthetech Inc., Albany, OR. The reactive side chains of the amino acids were masked with one of the following groups: Cys, 4-methylbenzyloxycarbonyl; His, 3-Bom; Lys, 2-chlorobenzyloxycarbonyl; Ser and Thr, *O*-benzyl; Tyr, *O*-2,6-dichlorobenzyl. All reagents and solvents were ACS grade or better and used without further purification.

Peptide Synthesis. The somatostatin antagonists were assembled on 4-methylbenzhydrylamine-functionalized, 1% cross-linked polystyrene resin (0.29 or 0.41 mequiv g^{-1}), in 0.25- or 0.5-mmol scale on a CS Bio Co. (San Carlos, CA; Model No. CS 136) or an Advanced ChemTech (model 200) synthesizer, using the following protocol: deblocking, 40% TFA (5 min, 19 min); DCM wash cycle (three washes); neutralization, 10% DIEA (1 min, 5 min); DMF wash cycle; DCM wash cycle (two washes); double coupling; first with 1,3-diisopropylcarbodiimide esters (3 equiv), 30 min in DCM; DCM wash (three washes); second coupling with preformed TBTU esters (3 equiv), 90 min in DMF, with a catalytic amount of DIEA; DMF wash (one wash); DCM wash (three washes). Coupling reactions were monitored qualitatively with the ninhydrin test.

Peptide Cleavage. The peptides were cleaved from the resin support with simultaneous side-chain deprotection by acidolysis using anhydrous hydrogen fluoride containing the scavengers anisole (~30% v/v) and dithiothreitol (~0.6% w/v) for 45 min at 0 °C. The peptides were cyclized in 90% acetic acid (~500 mL) with a slight excess of I_2 (15 min). Excess I_2 was then removed by the addition of ascorbic acid.

Purification. The crude peptides were purified by preparative RP-HPLC on C-18 bonded silica gel using axial compression columns (Dynamax-300 Å, 5 or 8 μm, 21.4 × 250 mm). A linear gradient elution system at a flow rate of 20 mL min^{-1} was employed: A, 0.1% TFA; B, 0.1% TFA in 80% MeCN; 20% B to 50% B at 1% min^{-1}. The separations were monitored by analytical RP-HPLC at 215 nm and TLC on silica gel plates (Merck F60) with ninhydrin visualization. The fractions containing the product were pooled, concentrated in vacuo, and subjected to lyophilization. Each peptide was obtained as a fluffy white powder of constant weight by lyophilization from aqueous acetic acid. The purity of the final peptides was assessed at 215 nm by analytical RP-HPLC in two systems. Analytical RP-HPLCs were recorded using a Vydac C-18 support (4.6 × 250 mm, 5 μm, 300 Å pore size, Liquid Separations Group). The two linear gradient systems were used at a flow rate of 1.5 mL min^{-1}: HPLC-1, A, 0.1% TFA; B, 0.1% TFA in 80% MeCN; 10% B to 70% B at 1% min^{-1}; and HPLC-2, C, 5% MeCN in TEAP (0.1 M, pH 3); D, 20% C in MeCN, 20% D to 80% D at 1% min^{-1}. Column eluent was monitored at 215 nm. The retention time and purity of each peptide were assessed by the Rainin Dynamax HPLC Method Manager. Each peptide was found to have a purity of ≥97%. The HPLC retention time results are given in Table 1.

Amino Acid Analysis. The peptides were hydrolyzed in vacuo (110 °C, 20 h) in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (Pierce). Amino acid analyses were performed on the hydrolysates following derivatization with *o*-phthaldialdehyde reagent (Sigma Chemical Co.) using an automatic HPLC system (Rainin Instrument Co.) fitted with a 100 × 4.6 mm, 3 μm C18 axial compression column with integral guard column (Microsorb AAAnalysis, Type O; Rainin Instrument Co.) The derivatized primary amino acids were eluted using a binary gradient of buffer A, 0.10 M sodium acetate containing 4.5% v/v methanol and 0.5% v/v tetrahydrofuran at pH 7.2, and buffer B, methanol. The gradient sequence: 0% A at 0 min, 35% A at 16.5 min, 90% A at 30 min, and 90% A at 33 min was used with a flow rate of 1.0 mL min^{-1} at ambient temperature. Eluent was monitored at 340 nm and integrated by the Dynamax HPLC Method Manager (Rainin). Standard retention times were as follows: Asp, 6.6 min; Arg, 19.9 min; Trp, 25.4 min; and Lys, 29.5 min. Each peptide produced the expected analytical results for the primary amino acids. Cysteine was not quantified. (Results not shown.)

Mass Spectrometry. The peptides were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass

spectrometry using a LaserMat 2000 mass spectrometer (Thermal Bioanalysis, San Jose, CA) using α-cyano-4-hydroxycinnamic acid as the matrix with Substance P (1348.7 Da) as an internal standard. In each case, the spectra consisted of a major $M - H^+$ ion peak for the internal standard, the expected analyte $M - H^+$ peak, and a few peaks associated with the matrix (<500 Da). The results are given in Table 1.

Antagonism of the in Vitro SRIF Inhibition of GH Release. Anterior pituitaries from adult male rats were collected and dispersed by a previously described trypsin/DNase method.[44] The dispersed cells were diluted with sterile-filtered Dulbecco's modified Eagle medium (MEM; Gibco Laboratories, Grand Island, NY), which was supplemented with 2.5% fetal calf serum (Gibco), 3% horse serum (Gibco), 10% fresh rat serum (stored on ice for no longer than 1 h) from the pituitary donors, 1% MEM nonessential amino acids (Gibco), gentamycin (10 ng mL^{-1}; Sigma), and nystatin (10,000 U mL^{-1}; Gibco). The cells were randomly plated at a density of approximately 200 000 cells/well (Costar cluster 24; Rochester Scientific Co., Rochester, NY). The plated cells were maintained in the above Dulbecco's medium in a humidified atmosphere of 95% air/5% CO_2 at 37 °C for 4−5 days. In preparation for a hormone challenge, the cells were washed with medium 199 (Gibco, 3 × 1 mL). Each dose of analogue (6 doses/plate) was tested in the presence of SRIF (1 nM) in triplicate wells in a total volume of 1 mL of medium 199 containing 1% BSA (fraction V; Sigma Chemical Co.). All wells contained GHRH(1−29)NH_2 (1 nM). A GHRH(1−29)NH_2 (1 nM)-stimulated control group and an SRIF (1 nM) with GHRH(1−29)NH_2 (1 nM)-inhibited control group were included on each cell culture plate. After the plates were incubated in an air/carbon dioxide atmosphere (95/5%, 3 h at 37 °C), the medium was removed and stored at −20 °C until assayed for hormone content. Growth hormone in media was measured by a standard double-antibody RIA using components generously supplied by the NHPP, NIDDK, NICHHD, and USDA. Antagonist IC_{50}'s versus SRIF (1 nM) were calculated using Sigmaplot (Jandel Scientific, San Rafael, CA). Values are expressed as the mean IC_{50} (nM) ± SEM from (*n*) separate dose−response curves and are given in Table 2.

Functional Expression of the Cloned Human Somatostatin Receptors. The genomic clones containing the human somatostatin receptors ($hsst_{1-5}$)[17,45−48] were kindly provided by Dr. Graeme I. Bell (University of Chicago). The $hsst_1$, $hsst_2$, $hsst_3$, $hsst_4$, and $hsst_5$ cDNAs were isolated as a 1.5-kb *Pst*I-*Xmn*I fragment, 1.7-kb *Bam*HI-*Hin*dIII fragment, 2.0-kb *Nco*I-*Hin*dIII fragment, 1.4-kb *Nhe*I-*Nde*I fragment, and 1.2-kb *Hin*dIII-*Xba*I fragment, respectively, each containing the entire coding region of the full-length receptors. These fragments were independently subcloned into the corresponding restriction endonuclease sites in the mammalian expression vector pCMV5, downstream from the human cytomegalovirus (CMV) promoter, to produce the expression plasmids pCMV5/$hsst_1$, pCMV5/$hsst_2$, pCMV5/$hsst_3$, pCMV5/$hsst_4$, and pCMV5/$hsst_5$. For transfection into CHO-K1 cells, a plasmid, pRSV-neo (American Type Culture Collection, Rockville, MD), carrying the neomycin mammalian cell selectable marker was added.

Receptor Expression and Transfection. Transfections were performed by the calcium phosphate method. CHO-K1 cells were maintained in α-MEM (Gibco) supplemented with 10% fetal calf serum and transfected with each of the expression plasmids using calcium phosphate precipitation. Clones that had inherited the expression plasmid were selected in α-MEM supplemented with 500 μg mL^{-1} Geneticin (G418; Gibco). Independent CHO-K1 clones were picked by glass-ring cloning and expanded in culture in the selective media. Membranes were prepared from the isolated clones, and hsst expression was initially assessed for binding with [^{125}I]Tyr^{11}-SRIF and [^{125}I]MK-678 (for sst_2).

Radioligand Binding Assays. Cell membranes of the five cell types were obtained from homogenates (Polytron setting 6, 15 s) of the corresponding CHO-K1 cells in ice-cold Tris-HCl (50 mM) and centrifuged (39000*g*, 10 min × 2), with an intermediate resuspension in fresh buffer. The final pellets were resuspended in Tris-HCl (10 mM) for assay. Aliquots of the membranes were incubated (30 min at 37 °C) with 0.05 nM [^{125}I]Tyr^{11}-SRIF (types 1, 3, 4, 5) or [^{125}I]MK-678 (type 2) in 50 nM HEPES (pH 7.4) containing BSA (10 mg mL^{-1}), $MgCl_2$ (5 mM), Trasylol (200 kIU mL^{-1}), bacitracin (0.02 mg mL^{-1}), and phenylmethanesulfonyl fluoride (0.02 mg mL^{-1}). The final assay volume was 0.3 mL, and incubations were terminated by rapid filtration through GF/C filters presoaked in 0.3% poly(ethylenimine) using a Brandel rapid filtration module. Each tube and filter were then washed with aliquots of cold buffer (3 × 5 mL). Specific binding was defined as the total radioligand bound minus that bound in the presence of 1.0 μM SRIF. The following total radioligand binding and nonspecific binding (nsb) values were typically obtained with these assay systems: $hsst_1$, 7000 cpm total versus 3500 cpm nsb; $hsst_2$, 9000 cpm total versus 1000 cpm nsb; $hsst_3$, 8000 cpm total versus 1000 cpm nsb; $hsst_4$, 6000 cpm total versus 3500 cpm nsb; and $hsst_5$, 7500 cpm total versus 3500 cpm nsb. The binding affinities expressed as K_i values ± SEM (nM) for each of the five receptor subtypes are given in Table 2.

Acknowledgment. We would like to thank Dr. Barry Morgan of Biomeasure Inc. for his useful discussions and comments, Ms. Etchie Yauger for her excellent support of this work in performing the purification of many peptides reported herein and amino acid analysis of all the peptides, Dr. Ning-Yi Jiang and Mr. Joseph Fuselier for the synthesis and purification of some of the peptides, Ms. Vienna Mackey for her assistance with the in vitro antagonist biological assays, and Ms. Karyn Van Buren for her expeditious administrative support.

References

(1) Brazeau, P.; Vale, W.; Burgus, R.; Ling, N.; Butcher, M.; Rivier, J.; Guillemin, R. Hypothalamic Polypeptide that Inhibits the Secretion of Immunoreactive Pituitary Growth Hormone. *Science* **1973**, *179*, 77−79.

(2) Mandarino, L.; Stenner, D.; Blanchard, W.; Nissen, S.; Gerich, J.; Ling, N.; Brazeau, P.; Bohlen, P.; Esch, F.; Guillemin, R. Selective Effects of Somatostatin −14, −25, and −28 on in vivo Insulin and Glucagon Secretion. *Nature* **1981**, *291*, 76−77.

(3) Bloom, S.; Mortimer, C.; Thorner, M.; Besser, G.; Hall, R.; Gomez-Pan, A.; Roy, U.; Russel, R.; Coy, D.; Kastin, A.; Schally, A. Inhibition of Gastrin and Gastric Acid Secretion by Growth Hormone Release-Inhibiting Hormone. *Lancet* **1974**, *2*, 1106−1109.

(4) Renaud, L.; Martin, J.; Brazeau, P. Depressant Action of TRH, LHRH and SRIF on Activity of Central Neurons. *Nature* **1975**, *255*, 233−235.

(5) Dodd, J.; Kelly, J. Is Somatostatin an Excitatory Transmitter in the Hippocampus? *Nature* **1978**, *273*, 674−677.

(6) Chesselet, M. F.; Reisine, T. Somatostatin Regulates Dopamine Release in Rat Striatal Slices and Cat Caudate Nuclei. *J. Neurosci.* **1983**, *3*, 232−236.

(7) Gothert, M. Somatostatin Selectively Inhibits Noradrenaline Release from Hypothalamic Neurons. *Nature* **1980**, *288*, 86−88.

(8) Havlicek, V.; Rezek, M.; Freisen, H. Somatostatin and Thyrotropin Releasing Hormone Central Effect on Sleep and Motor Systems. *Pharmacol. Biochem. Behav.* **1976**, *4*, 455−459.

(9) Schettini, G. Brain Somatostatin: Receptor-Coupled Transducing Mechanisms and role in Cognitive Functions. *Pharmacol. Res.* **1991**, *23*, 203−215.

(10) Reisine, T.; Bell, G. I. Molecular Properties of Somatostatin Receptors. *Neuroscience* **1995**, *67*, 777−790.

(11) Reisine, T.; Bell, G. I. Molecular Biology of Somatostatin Receptors. *Endocrine Rev.* **1995**, *16*, 427−442.

(12) Coy, D. H.; Taylor, J. E. Receptor-Specific Somatostatin Analogues: Correlations with Biological Activity. *Metabolism* **1996**, *34* (Suppl. 1), 21−23.

(13) Raynor, K.; Murphy, W. A.; Coy, D. H.; Taylor, J. E.; Moreau, J.-P.; Yasuda, K.; Bell, G. I.; Reisine, T. Cloned Somatostatin Receptors: Identification of Subtype-Selective Peptides and Demonstration of High Affinity Binding of Linear Peptides. *Mol. Pharmacol.* **1993**, *43*, 838−844.

(14) Rossowski, W. J.; Coy, D. H. Specific Inhibition of Rat Pancreatic Insulin and Glucagon Release by Receptor-Selective Somatostatin Analogues. *Biochem. Biophys. Res. Commun.* **1994**, *205*, 341−346.

(15) Rossowski, W. J.; Gu, Z. F.; Akarca, U. S.; Jensen, R. T.; Coy, D. H. Characterization of Somatostatin Receptor Subtypes Controlling Rat Gastric Acid and Pancreatic Amylase Release. *Peptides* **1994**, *15*, 1421−1424.
(16) Murthy, K. S.; Coy, D. H.; Makhlouf, G. M. Somatostatin Receptor-Mediated Signaling in Smooth Muscle − Activation of Phospholipase C-β3 by Gβgamma and Inhibition of Adenyl Cyclase by GαiIGαo. *J. Biol. Chem*. **1996**, *271*, 23458−23463.
(17) O'Carroll, A.-M.; Raynor, K.; Lolait, S. J.; Reisine, T. Characterization of Cloned Human Somatostatin Receptor SSTR5. *Mol. Pharmacol*. **1994**, *46*, 291−298.
(18) Rossowski, W.; Coy, D. H. Potent Inhibitory effects of a Type Four Receptor Selective Somatostatin Analogue on Rat Insulin Release. *Biochem. Biophys. Res. Commun*. **1993**, *197*, 366−371.
(19) Fries, J. L.; Murphy, W. A.; Sueiras-Diaz, J.; Coy, D. H. Somatostatin Antagonist Analogue Increases GH, Insulin and Glucagon Release in the Rat. *Peptides* **1982**, *3*, 811−814.
(20) Spencer, G. S. G.; Hallett, K. G. Somatostatin Antagonist Analogue Stimulates Growth in Rats. *Life Sci*. **1985**, *37*, 27−30.
(21) Hocart, S. J.; Jain, R.; Murphy, W. A.; Taylor, J. E.; Morgan, B.; Coy, D. H. Potent Antagonists of Somatostatin, Synthesis and Biology. *J. Med. Chem*. **1998**, *41*, 1146−1154.
(22) Bass, R. T.; Buckwalter, B. L.; Patel, B. P.; Pausch, M. H.; Price, L. A.; Strnad, J.; Hadcock, J. R. Identification and Characterization of Novel Somatostatin Antagonists. *Mol. Pharmacol*. **1996**, *50*, 709−715.
(23) Bass, R. T.; Buckwalter, B. L.; Patel, B. P.; Pausch, M. H.; Price, L. A.; Strnad, J.; Hadcock, J. R. Identification and Characterization of Novel Somatostatin Antagonists, Erratum. *Mol. Pharmacol*. **1997**, *51*, 170.
(24) Coy, D. H.; Murphy, W. A.; Rossowski, W. J.; Hocart, S. J.; Jain, R.; Fuselier, J.; Taylor, J. E. Somatostatin Receptor Antagonists based an a mixed Neuromedin B Antagonist/Somatostatin Agonist. In *Peptides: Frontiers of Peptide Science, Proceedings of the Fifteenth American Peptide Symposium*; Tam, J. P., Kaumaya, P. T. P., Eds.; Kluwer: Dordrecht, The Netherlands, 1999; pp 526−529.
(25) Brady, S. F.; Nutt, R. F.; Holly F. W.; Paleveda, W. J.; Strachan, R. G.; Bergstrand, S. J.; Veber, D. F.; Saperstein, R. Synthesis and Biological Activity of Somatostatin Analogues of Reduced Ring Size. In *Peptides: Synthesis, Structure, Function, Proceedings of the Seventh American Peptide Symposium*; Rich, D. H., Gross, E., Eds.; Pierce Chemical Co.: New York, 1981; pp 653−656.
(26) Veber, D. F. Design of a Highly Active Cyclic Hexapeptide Analogue of Somatostatin. In *Peptides: Synthesis, Structure, Function. Proceedings of the Seventh American Peptide Symposium*; Rich, D. H., Gross, E., Eds.; Pierce Chemical Co.: New York, 1981; pp 685−694.
(27) Veber, D. F.; Saperstein, R.; Nutt, R. F.; Freidinger, R. M.; Brady, S. F.; Curley, P.; Perlow, D. S.; Paleveda, W. J.; Colton, C. D.; Zacchei, A. G.; Tocco, D. J.; Hoff, D. R.; Vandlen, R. L.; Gerich, J. E.; Hall, L.; Mandarino, L.; Cordes, E. H.; Anderson, P. S.; Hirschmann, R. A Super Active Cyclic Hexapeptide Analogue of Somatostatin. *Life Sci*. **1984**, *34*, 1371−137.
(28) Veber, D. F. Design and Discovery in the Development of Peptide Analogues. In *Peptides: Chemistry and Biology. Proceedings of the Twelfth American Peptide Symposium*; Smith, J. A., Rivier, J. E., Eds.; ESCOM: Leiden, 1992; pp 3−14.
(29) Murphy, W. A.; Heiman, M. L.; Lance, V.; Mezo, I.; Coy, D. H. Octapeptide Analogues of Somatostatin Exhibiting Greatly Enhanced in vivo and in vitro Inhibition of Growth Hormone Secretion in the Rat. *Biochem. Biophys. Res. Commun*. **1985**, *132*, 922−928.
(30) Freidinger, R. M.; Schwenk Perlow, D.; Randall, W. C.; Saperstein, R.; Arison, B. H.; Veber, D. F. Conformational Modifications of Cyclic Hexapeptide Somatostatin Analogues. *Int. J. Pept. Protein Res*. **1984**, *23*, 142−150.
(31) Ösapay, G.; Prokai, L.; Ho-Seung, K.; Medzihradszky, K. F.; Coy, D. H.; Liapakis, G.; Reisine, T.; Melacini, G.; Zhu, Q.; Wang, S. H.-H.; Mattern, R.-H.; Goodman, M. Lanthionine-Somatostatin Analogues: Synthesis, Characterization, Biological Activity, and Enzymatic Stability Studies. *J. Med. Chem*. **1997**, *40*, 2241−2251.
(32) Chen, L.; Skinner, S. R.; Gordon, T. D.; Taylor, J. E.; Barany, G.; Morgan, B. A. Isolation, Characterization and Synthesis of a Trisulfide Related to the Somatostatin Analogue Lanreotide. In *Peptides: Frontiers of Peptide Science, Proceedings of the Fifteenth American Peptide Symposium*; Tam, J. P., Kaumaya, P. T. P., Eds.; Kluwer: Dordrecht, The Netherlands, 1999; pp 275−276.
(33) Thurieau, C.; Janiak, P.; Krantic, S.; Guyard, C.; Pillon, A.; Kucharczyk, N.; Vilaine, J.-P.; Fauchère, J.-L. A New Somatostatin Analogue with Optimized Ring Size Inhibits Neointimal Formation Induced by Balloon Injury in Rats Without Altering Growth Hormone Release. *Eur. J. Med. Chem*. **1995**, *30*, 115−122.
(34) Van Binst, G.; Tourwé, D. Backbone Modifications in Somatostatin Analogues: Relation Between Conformation and Activity. *Pept. Res*. **1992**, *5*, 8−13.
(35) Rossowski, W. J.; Cheng, B.-L.; Jiang, N.-Y.; Coy, D. H. Examination of Somatostatin Involvement in the Inhibitory Action of GIP, GLP-1, Amylin and Adrenomedullin on Gastric Acid Release using a new SRIF Antagonist Analogue. *Br. J. Pharmacol*. **1998**, *125*, 1081−1087.
(36) The model diastereoisomeric peptides were H-Nal-D&LCys-His-DTrp-Lys-Val-Cys-Nal-NH_2 and H-DNal-Cys-His-DTrp-Lys-Val-Cys-D&LNal−NH_2 which had retention times in the TFA HPLC system of LCys, 30.3; DCys, 30.9; LNal, 36.6; DNal, 37.8 min, respectively.
(37) Binding data not shown.
(38) Reisine, T.; Bell, G. I. Molecular Properties of Somatostatin Receptors *Neuroscience* **1995**, *67*, 777−790.
(39) Liapakis, G.; Hoeger, C.; Rivier, J.; Reisine, T. Development of a Selective Agonist at the Somatostatin Receptor Subtype SSTR1. *J. Pharmacol. Exp. Ther*. **1996**, *276*, 1089−1094.
(40) Hoyer, D.; Bell, G. I.; Berelewitz, M.; Epelbaum, J.; Feniuk, W.; Humphrey, P. P. A.; O'Carroll, A.-M.; Patel, Y. C.; Schonbrunn, A.; Taylor, J. E.; Reisine, T. Classification and Nomenclature of Somatostatin Receptors. *Trends Pharmacol. Sci*. **1995**, *16*, 86−88.
(41) Bruno, J.-F.; Xu, Y.; Song, J.; Berelowitz, M. Molecular Cloning and Functional Expression of a Novel Brain Specific SRIF Receptor. *Proc. Natl. Acad. Sci. U.S.A*. **1992**, *89*, 11151−11155.
(42) O'Carroll, A.-M.; Lolait, S. J.; Konig, M.; Mahan, L. C. Molecular Cloning and Expression of a Pituitary SRIF Receptor with Preferential Affinity for SRIF-28. *Mol. Pharmacol*. **1992**, *42*, 939−946.
(43) IUPAC−IUB Commission of Biochemical Nomenclature (CBN) Symbols for Amino-Acid Derivatives and Peptides, Recommendations 1971. *Eur. J. Biochem*. **1972**, *27*, 201−207.
(44) Murphy, W. A.; Taylor, J.; Moreau, J.-P.; Coy, D. H. Novel Heptapeptide Somatostatin Analogue Displays Antitumor Activity Independent of Effects on Growth Hormone Secretion. *Pept. Res*. **1989**, *2*, 128−132.
(45) Yamada, Y.; Post, S. R.; Wang, K.; Tager, H. S.; Bell, G. I.; Seino, S. Cloning and Functional Characterization of a Family of Human and Mouse Somatostatin Receptors Expressed in Brain, Gastrointestinal Tract and Kidney. *Proc. Natl. Acad. Sci. U.S.A*. **1992,** *89*, 251−255.
(46) Yasuda, K.; Rens-Domiano, S.; Breder, C. D.; Law, S. F.; Sapel, C. B.; Reisine, T.; Bell, G. I. Cloning of a Novel Somatostatin Receptor, SSTR-3, Coupled to Adenyl Cyclase. *J. Biol. Chem*. **1992**, *267*, 20422−20428.
(47) Yamada, Y.; Reisine, T.; Law, S. F.; Ihara, Y.; Kubota, A.; Kagimoto, S.; Seino, M.; Seino, Y.; Bell, G. I.; Seino, S. Somatostatin Receptors, an Expanding Gene Family: Cloning and Functional Characterization of Human SSTR3, a Protein Coupled to Adenylate Cyclase. *Mol. Pharmacol*. **1992,** *42*, 2136−2142.
(48) Rohrer, L.; Raulf, F.; Bruns, C.; Buettner, R.; Hofstaedter, F.; Schule, R. Cloning and Characterization of a Fourth Human Somatostatin Receptor. *Proc. Natl. Acad. Sci. U.S.A*. **1993**, *90*, 4196−4200.
(49) Taylor, J. E. Personal communication.

JM9806289