Novel sst_4-Selective Somatostatin (SRIF) Agonists. 3. Analogues Amenable to Radiolabeling

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After our discovery that H-c[Cys-Phe-Phe-DNal-Lys-Thr-Phe-Cys]-OH (ODN-8) had high affinity and marginal selectivity for human sst_3 (part 2 of this series: Erchegyi et al. *J. Med. Chem.*, preceding paper in this issue)[11] and that H-c[Cys-Phe-Phe-DTrp-Lys-Thr-Phe-Cys]-OH (ODT-8, **3**) had high affinity and marginal selectivity for human sst_4, that H-c[Cys-Phe-Tyr-D-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH had high affinity for all sst's except for sst_1, and that H-c[Cys-Phe-Tyr-L-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH had high affinity for sst_4 (IC_{50} = 2.1 nM), with more than 50-fold selectivity toward the other receptors (parts 1 and 2 of this series: Rivier et al. and Erchegyi et al. *J. Med. Chem.*, preceding papers in this issue), we found H-c[Cys-Phe-Phe-Trp-Lys-Thr-Phe-Cys]-OH (OLT-8, **2**), H-c[Cys-Phe-Phe-L-*threo*-β-MeTrp-Lys-Thr-Phe-Cys]-OH (**4**) and H-c[Cys-Phe-Phe-D-*threo*-β-MeTrp-Lys-Thr-Phe-Cys]-OH (**5**) to have very high affinity for sst_4 (IC_{50} = 0.7, 1.8, and 4.0 nM, respectively) and 5- to 10-fold selectivity versus the other sst's. From earlier work, we concluded that an L-amino acid at position 8 and a tyrosine or 4-aminophenylalanine substitution at position 7 may lead to high sst_4 selectivity. In fact, [Tyr^7]-**2** (**6**) and [Tyr^7]-**3** (**7**) show ca. 5-fold selectivity for sst_4, and [Aph^7]-**2** (**8**) and [Aph^7]-**3** (**9**) have high sst_4 affinity (IC_{50} = 1.2 and 0.88 nM, respectively) and selectivity, suggesting that indeed an L-residue at position 8 will direct selectivity toward sst_4. Unexpectedly, [Ala^7]-**2** (**10**) and [Ala^7]-**3** (**11**) have very high sst_4 affinity (IC_{50} = 0.84 and 0.98 nM, respectively) and selectivity (>600- and 200-fold, respectively). The combination of Tyr^2 and $DTrp^8$ in analogues **14** and **22** did not affect the affinity of the analogues for sst_4 (IC_{50} = 1.2 and 1.1 nM, respectively) but resulted in loss of selectivity, whereas the combination of Tyr^2 and $LTrp^8$ in H-Tyr-c[Cys-Phe-Aph-Trp-Lys-Thr-Phe-Cys]-OH (**13**) and H-Tyr-c[Cys-Phe-Ala-Trp-Lys-Thr-Phe-Cys]-OH-(**19**) retained high affinity (IC_{50} = 1.9 and 1.98 nM, respectively) and sst_4 selectivity (>50 and >250, respectively). Interestingly, the same substitutions at positions 2 and 7, with L-*threo*-β-MeTrp at position 8, yielded a much less selective analogue (**20**). Carbamoylation of the N-terminus of most of these analogues resulted in slightly improved affinity, selectivity, or both. Other amino acid substitutions in this series, such as those with Amp (**25**, **26**), Orn (**27**), or IAmp (**29**) at position 7, were also tolerated but with a 2- to 3-fold loss of affinity and concomitant loss of selectivity. Analogous peptides with a tyrosine at position 11 (**31**−**36**) were less selective than the corresponding peptides with a tyrosine at position 2. Several analogues in this series compared favorably with the non-peptide L-803,087 (**37**) in terms of affinity and selectivity. Analogues **8**, **10**, and **21** potently inhibited the forskolin-stimulated cAMP production in sst_4-transfected cells, therefore acting as full agonists. Cold monoiodination of **19** yielded **21**, with retention of high sst_4 selectivity and affinity (IC_{50} = 3.5 nM). ^{125}Iodinated **19** selectively binds to sst_4-transfected cells but not to sst_{1-3}- or sst_5-transfected cells. Binding in sst_4-transfected cells was completely displaced by SRIF-28 or the sst_4-selective L-803,087.

Introduction

As stated in the first paper in this series,[1] the general aim of our laboratories has been to study the tissue distribution, mechanism of action, and physiological role as well as pharmacological effects of the hypothalamic releasing hormones in the endocrine, exocrine, and nervous systems. To achieve these goals, we must successfully design peptide analogues that are (1) potent and highly receptor-selective, (2) potent and highly receptor-selective peptide tracers for binding assays and scintigraphy studies, (3) potent and highly receptor-selective peptide agonists, and (4) antagonists for in vitro (mechanistic) and in vivo (functional/physiological) studies. It is a particular challenge in the case of somatostatin with five identified receptors.[2−9] Progress has been made recently toward achieving these goals: we have described analogues that bind to sst_1 receptors that fulfill criteria 1−3,[10] analogues that bind to sst_3 that fulfill criteria 1, 2, and 4,[11] and analogues that bind to sst_4 that fulfill criteria 1 and 3.[1,12,13] Here we describe our approach to the design of an analogue that binds to

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sst_4 and fulfills criteria 2 in addition to criteria 1 and 3, i.e., that is a potent sst_4-selective agonist tracer.

In the preceding papers,[12,13] we described the chemical and biological characterization of a series of sst_4-selective agonists with H-c[Cys-Phe-Tyr-L-*threo*-β-Me2-Nal-Lys-Thr-Phe-Cys]-OH, having >100-fold selectivity for sst_4 over the other four sst's while retaining sst_4 binding affinity (IC_{50} = 2.7 nM) comparable to that of SRIF-28 (IC_{50} = 3.8 nM). Unfortunately, monoiodination of this analogue resulted in significant loss of affinity (IC_{50} = 21 nM). Although we had achieved one of our goals to generate a potent sst_4-selective ligand (characterized to be an agonist), we were unable to develop it as a tracer for binding or localization studies.

Subsequent to the discovery that H-c[Cys-Phe-Tyr-DNal-Lys-Thr-Phe-Cys]-OH (ODN-8) had high affinity and marginal selectivity for human sst_3,[11] that ODT-8 (**3**) had high affinity and marginal selectivity for human sst_4,[11] and the study presented in the accompanying papers,[1,12,13] we now find that OLT-8 (**2**) has very high affinity for sst_4 (IC_{50} = 0.7 nM) and 5- to 10-fold selectivity versus the other sst's. This is in keeping with a similar observation published recently in Lewis et al.'s alanine scan of SRIF-NH_2.[14] In summary, although circuitous, the rationale that led to the identification of position 7 as important for sst_4 selectivity came from several independent observations. First, H-c[Cys-Phe-LAgl(N^{β}Me,benzoyl)-DTrp-Lys-Thr-Phe-Cys]-OH, with IC_{50}'s for sst_{1-5} = >1000, 460, 447, 5.2, and 768 nM, respectively, pointed to the importance of residue 7.[1] Second, the relative lack of selectivity of H-c[DCys-Phe-Tyr-D-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH (IC_{50}'s for sst_{1-5} = 545, 12, 14, 0.53, and 27 nM, respectively) versus H-c[Cys-Phe-Tyr-L-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH (IC_{50}'s for sst_{1-5} = >10K, 339, 664, 3.5, and 668, respectively)[12,13] suggested a critical role for an L-configuration rather than D at position 8. Third, at least in one series,[1] substitution of $LCys^3$ by $DCys^3$ did not bear significantly on either selectivity or activity since the representative analogues H-c[Cys-Phe-Tyr-L-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH and H-c[DCys-Phe-Tyr-L-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH were sst_4-selective and were agonists in a cAMP accumulation test.[12,13] We concluded that an L-amino acid at position 8 and an optimized substitution at position 7 (such as tyrosine or 4-aminophenylalanine, Aph) may lead to increased sst_4 selectivity and used this lead for further SAR studies.

Results and Discussion

All analogues shown in Table 1 were synthesized either manually or automatically on a chloromethylated resin using the Boc strategy and diisopropylcarbodiimide (DIC) for amide bond formation and trifluoroacetic acid (TFA) for Boc removal. The peptide resins were treated with hydrogen fluoride (HF) in the presence of scavengers to liberate the fully deblocked crude linear peptides. Most amino acid derivatives used are commercially available; the threo and erythro isomers of β-methyltryptophan were prepared according to the method of Snyder.[15] Cyclization of the cysteines was mediated by iodine in an acidic milieu. Purification was carried out using multiple HPLC steps[16] and characterized by HPLC,[16] capillary zone electrophoresis (CZE),[17] and mass spectrometry. The measured masses obtained using MALDI-MS were within 100 ppm of those calculated for the protonated molecule ions.

The compounds were tested for their ability to bind to 20-μm-thick cryostat sections of a membrane pellet of cells transfected with the five human sst receptor subtypes. For each of the tested compounds, complete displacement experiments with the universal somatostatin radioligand [Leu^8,$DTrp^{22}$,$^{125}ITyr^{25}$]SRIF-28 (30 000 cpm/100 μL), using increasing concentrations of the unlabeled peptide ranging from 0.1 to 1000 nM, were performed. The unlabeled SRIF-28 was run in parallel using the same increasing concentrations as control. IC_{50} values were calculated after quantification of the data using a computer-assisted image-processing system as described previously.[18,19] Tissue standards that contain known amounts of isotope, cross-calibrated to tissue-equivalent ligand concentrations, were used for quantitation.[20] The advantages of the present method using receptor autoradiography with sectioned cell pellets compared to binding on cell homogenates are, in addition to an economy on cells and a great flexibility, the greater inter-assay reliability and reproducibility, since sections of the same embedded pellet can be used for successive experiments. As a minor disadvantage, IC_{50} values are somewhat higher than those obtained in the homogenate binding assay.[18] The most potent and selective analogues were then evaluated for their agonist/antagonist properties, measuring inhibition of cyclic adenosine monophosphate (cAMP) production. The ^{125}monoiodinated form of the best sst_4 analogues was also used to label sst_4-transfected cells.

Considering the result of several observations described in the preceding papers[1,12,13] and outlined below, we felt compelled to re-examine 20-year-old biological results (based on the ability of somatostatin and analogues to inhibit spontaneous secretion of growth hormone by pituitary cells in culture)[21] in light of our present ability to test these analogues for their affinity at each of the five identified somatostatin receptors. First, we showed that H-c[Cys-Phe-Phe-DNal-Lys-Thr-Phe-Cys]-OH (ODN-8) had high affinity and marginal selectivity for human sst_3,[11] and that ODT-8 (**3**) had high affinity and marginal selectivity for human sst_4. This suggested that a DTrp substitution might be more favorable than a DNal at position 8 in the design of an sst_4-selective analogue, although we have already shown that DNal-based analogues could be designed to be highly selective for that receptor.[12,13] We then concluded, on the basis of the observation that H-c[Cys-Phe-Tyr-D-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH had high affinity for all sst's except for sst_1 and that H-c[Cys-Phe-Tyr-L-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH had high affinity for sst_4 (IC_{50} = 2.1 nM), with more than 50-fold selectivity toward the other receptors, that an L-configuration at position 8 may have a profound effect on directing selectivity for sst_4.[12,13] This was confirmed when we tested OLT-8 (**2**) with very high affinity for sst_4 (IC_{50} = 0.7 nM) and 5- to 10-fold selectivity versus the other sst's. Interestingly, the binding affinity profile of **4** and **5** is not significantly different than that of **2** and **3**, respectively, suggesting that the additional conformational constraint brought about by β-methylation at position 8 was ineffective in directing selectiv-

Table 1. Physicochemical Properties and sst$_{1-5}$ Binding Affinities (IC_{50}, nM) of sst$_4$-Selective Analogues and Control Peptides

		purity (%)		MS[c]		IC_{50} (nM)[d]				
no.	compd	HPLC[a]	CZE[b]	M(mono) calcd	MH$^+$(mono) obsd	sst$_1$	sst$_2$	sst$_3$	sst$_4$	sst$_5$
1	somatostatin-28, SRIF-28	98	98	3146.5	3147.7	3.2 ± 0.2 (36)	2.3 ± 0.1 (37)	3.5 ± 0.2 (36)	2.5 ± 0.1 (35)	2.4 ± 0.2 (34)
2	H-c[Cys-Phe-Phe-Trp-Lys-Thr-Phe-Cys]-OH (OLT-8)	97	98	1078.44	1079.4	5.3 ± 0.7 (3)	130 ± 65 (3)	13 ± 0.7 (3)	0.7 ± 0.3 (3)	14 ± 4.1 (3)
3	H-c[Cys-Phe-Phe-D-Trp-Lys-Thr-Phe-Cys]-OH (ODT-8)	>94	98	1078.44	1079.2	27 ± 3.4 (4)	41 ± 8.7 (6)	13 ± 3.2 (4)	1.8 ± 0.7 (4)	46 ± 27 (3)
4	H-c[Cys-Phe-Phe-L-*threo*-β-MeTrp-Lys-Thr-Phe-Cys]-OH	98	98	1092.46	1093.4	21 (17; 25)	27 (27, 26)	330 (210; 449)	1.8 (2.7; 0.9)	221 (190; 251)
5	H-c[Cys-Phe-Phe-D-*threo*-β-MeTrp-Lys-Thr-Phe-Cys]-OH	90	90	1092.46	1093.5	49 (40; 58)	40 (24; 54)	53 (44; 61)	4 (5.2; 2.9)	42 (28; 52)
6	H-c[Cys-Phe-Tyr-Trp-Lys-Thr-Phe-Cys]-OH	97	96	1094.44	1095.4	27 ± 6.8 (3)	54 ± 16 (3)	22 ± 1.7 (3)	1.3 ± 0.1 (3)	63 ± 7 (3)
7	H-c[Cys-Phe-Tyr-D-Trp-Lys-Thr-Phe-Cys]-OH	>98	98	1094.44	1095.0	117 ± 37 (3)	25.7 ± 6 (3)	35.7 ± 8 (3)	1.8 ± 0.7 (3)	20.3 ± 2.9 (3)
8	H-c[Cys-Phe-Aph-Trp-Lys-Thr-Phe-Cys]-OH	98	97	1093.45	1094.3	213 ± 19 (4)	347 ± 61 (3)	>1000 (3)	1.2 ± 0.1 (4)	1000 ± 91 (4)
9	H-c[Cys-Phe-Aph-D-Trp-Lys-Thr-Phe-Cys]-OH	>98	98	1093.45	1094.3	450 ± 135 (3)	71 ± 2.1 (3)	271 ± 120 (3)	0.88 ± 0.3 (3)	30 ± 7.4 (3)
10	H-c[Cys-Phe-Ala-Trp-Lys-Thr-Phe-Cys]-OH	>98	>98	1002.41	1003.4	>1000 (3)	807 ± 146 (3)	750 ± 278 (3)	0.84 ± 0.2 (3)	633 ± 186 (3)
11	H-c[Cys-Phe-Ala-D-Trp-Lys-Thr-Phe-Cys]-OH	93	94	1002.41	1003.5	>1000 (3)	183 ± 18 (3)	897 ± 103 (3)	0.98 ± 0.1 (4)	199 ± 56 (4)
12	H-Tyr-c[Cys-Phe-Phe-Trp-Lys-Thr-Phe-Cys]-OH	99	99	1241.50	1242.4	13 (14; 11)	179 (150; 207)	57 (66; 47)	1.6 (1; 2.1)	19 (18; 20)
13	H-Tyr-c[Cys-Phe-Aph-Trp-Lys-Thr-Phe-Cys]-OH	98	>98	1256.51	1257.5	270 ± 39 (4)	260 ± 20 (3)	135 ± 25 (4)	1.9 ± 0.3 (4)	663 ± 157 (4)
14	H-Tyr-c[Cys-Phe-Aph-D-Trp-Lys-Thr-Phe-Cys]-OH	99	98	1256.51	1257.6	517 ± 159 (3)	56 ± 5.8 (3)	263 ± 97 (3)	1.2 ± 0.3 (3)	34 ± 8.6 (3)
15	H_2N−CO-c[Cys-Phe-Aph-Trp-Lys-Thr-Phe-Cys]-OH	91	92	1136.46	1137.4	650 ± 115 (3)	>1000 (3)	780 ± 62 (3)	1.5 ± 0.07 (3)	>1000 (3)
16	H_2N−CO-c[Cys-Phe-Aph-D-Trp-Lys-Thr-Phe-Cys]-OH	>98	>98	1136.46	1137.2	840 ± 101 (4)	120 ± 15 (3)	650 ± 161 (3)	1.1 ± 0.2 (3)	103 ± 15 (3)
17	H_2N−CO-Tyr-c[Cys-Phe-Aph-Trp-Lys-Thr-Phe-Cys]-OH	92	92	1299.52	1300.6	347 ± 120 (3)	550 ± 276 (3)	210 ± 36 (3)	1.2 ± 0.3 (3)	317 ± 92 (3)
18	H_2N-CO-(m-I)-Tyr-c[Cys-Phe-Aph-Trp-Lys-Thr-Phe-Cys]-OH	97	97	1425.43	1426.4	>1000 (2)	>1000 (2)	325 (500; 150)	8.8 ± 1.3 (4)	990 (480; 1500)
19	H-Tyr-c[Cys-Phe-Ala-Trp-Lys-Thr-Phe-Cys]-OH	99	99	1165.47	1166.3	>1000 (4)	622 ± 85 (4)	624 ± 213 (4)	2.0 ± 0.5 (4)	692 ± 166 (4)
20	H-Tyr-c[Cys-Phe-Phe-L-*threo*-β-Me-Trp-Lys-Thr-Phe-Cys]-OH	96	98	1179.49	1180.5	88 (88; 87)	48 (18; 77)	116 (75;156)	0.9 (1.2; 0.6)	393 (350; 435)
21	H-(m-I)-Tyr-c[Cys-Phe-Ala-Trp-Lys-Thr-Phe-Cys]-OH	99	99	1291.38	1292.3	>1000 (2)	>1000 (2)	1025 (550; 1500)	3.5 ± 0.8 (4)	>1000 (2)
22	H-Tyr-c[Cys-Phe-Ala-D-Trp-Lys-Thr-Phe-Cys]-OH	98	97	1165.47	1166.4	330 ± 112 (3)	57 ± 1.7 (3)	347 ± 120 (3)	1.1 ± 0.2 (3)	51 ± 20 (3)
23	H-Tyr-c[Cys-Phe-Ala-D-*threo*-β-Me-Trp-Lys-Thr-Phe-Cys]-OH	99	97	1179.49	1180.5	>1000 (400; 2792)	46 (34; 57)	381 (110; 652)	5.6 (5.5; 5.6)	629 (410; 847)
24	H_2N-CO-Tyr-c[Cys-Phe-Ala-Trp-Lys-Thr-Phe-Cys]-OH	84	96	1208.49	1209.5	>1000 (3)	700 ± 92 (3)	980 ± 73 (3)	2.4 ± 0.5 (3)	>1000 (3)
25	H-Tyr-c[Cys-Phe-Amp-Trp-Lys-Thr-Phe-Cys]-OH	97	98	1270.53	1271.6	>1000 (2)	259 (250; 267)	326 (319−332)	2.4 ± 0.2 (3)	342 (260; 424)
26	H_2N-CO-Tyr-c[Cys-Phe-Amp-Trp-Lys-Thr-Phe-Cys]-OH	91	91	1313.55	1314.5	>1000 ± 50 (3)	118 ± 42 (3)	197 ± 48 (3)	3.2 ± 0.6 (3)	247 ± 27 (3)
27	H_2N-CO-Tyr-c[Cys-Phe-Orn-Trp-Lys-Thr-Phe-Cys]-OH	96	98	1251.52	1252.5	99 (60; 137)	561 (250; 872)	>1000 (420; 3722)	2.1 (2.2; 2.0)	458 (550; 365)
28	H_2N-CO-Tyr-c[Cys-Phe-Amp-D-Trp-Lys-Thr-Phe-Cys]-OH	93	99	1313.55	1314.5	>1000 (3)	29 ± 22 (3)	>1000 (3)	2.6 ± 0.2 (3)	50 ± 5 (3)
29	H_2N-CO-Tyr-c[Cys-Phe-IAmp-Trp-Lys-Thr-Phe-Cys]-OH	>98	96	1355.58	1356.6	323 ± 63 (3)	130 ± 32 (3)	110 ± 17 (4)	2.3 ± 0.5 (4)	397 ± 152 (3)
30	H_2N-CO-Tyr-c[Cys-Phe-IAmp-D-Trp-Lys-Thr-Phe-Cys]-OH	98	>98	1355.58	1356.6	397 ± 165 (3)	12 ± 1.5 (3)	>1000 (3)	2.3 ± 0.4 (3)	84 ± 23 (3)
31	H-c[Cys-Phe-Phe-Trp-Lys-Thr-Tyr-Cys]-OH	99	98	1094.44	1095.4	6.5 ± 1.8 (3)	43 ± 13 (3)	10 ± 1.7 (3)	1.3 ± 0.2 (3)	24 ± 6.5 (3)
32	H-c[Cys-Phe-Phe-D-Trp-Lys-Thr-Tyr-Cys]-OH	99	98	1094.44	1095.3	10.3 ± 0.9 (3)	15.5 ± 6 (3)	8.2 ± 4.4 (3)	0.51 ± 0.1 (3)	4.8 ± 0.33 (3)
33	H_2N-CO-c[Cys-Phe-Phe-D-Trp-Lys-Thr-Tyr-Cys]-OH	96	99	1137.44	1138.8	55 (43; 67)	52 (35; 68)	16 (9.5; 22)	1.0 (1.1; 0.9)	7.6 (9.5; 5.7)
34	H-c[Cys-Phe-Aph-Trp-Lys-Thr-Tyr-Cys]-OH	99	98	1109.45	1110.36	327 ± 37 (3)	170 ± 25 (3)	247 ± 86 (3)	1.1 ± 0.2 (3)	240 ± 45 (3)
35	H_2N-CO-c[Cys-Phe-Aph-Trp-Lys-Thr-Tyr-Cys]-OH	86	90	1152.45	1153.44	757 ± 94 (3)	>1000 (3)	587 ± 150 (3)	3 ± 0.4 (3)	557 ± 70 (3)
36	H_2N-CO-c[Cys-Phe-Aph-D-Trp-Lys-Thr-Tyr-Cys]-OH	90	98	1152.45	1153.4	>1000 (3)	227 ± 108 (3)	623 ± 340 (3)	4.3 ± 2.4 (3)	110 ± 12 (3)
37	L-803,087 (Merck)[26]					>1000 (4)	>10K (4)	>10K (4)	4.2 ± 1.9 (3)	>10K (4)

[a] Percent purity determined by HPLC using buffer system: A = TEAP (pH 2.5) and B = 60% CH_3CN/40% A, with a gradient slope of 1% B/min, at flow rate of 0.2 mL/min on a Vydac C_{18} column (0.21 × 15 cm, 5 μm particle size, 300 Å pore size). Detection at 214 nm. [b] Capillary zone electrophoresis (CZE) was done using a Beckman P/ACE system 2050 controlled by an IBM Personal System/2 model 50Z and using a ChromJet integrator; field strength of 15 kV at 30 °C. Mobile phase: 100 mM sodium phosphate (85:15, H_2O:CH_3CN), pH 2.50, on a Supelco P175 capillary (363 μm o.d. × 75 μm i.d. × 50 cm length). Detection at 214 nm. [c] The calculated *m*/*z* of the monoisotope compared with the observed $[M + H]^+$ monoisotopic mass. [d] The IC_{50} values (nM) were derived from competitive radioligand displacement assays reflect the affinities of the analogues for the cloned somatostatin receptors using the nonselective ^{125}I-[Leu^8,D-Trp^{22},Tyr^{25}]SRIF-28, as the radioligand. Mean value ± SEM when $N \geq 3$ (shown in parentheses). Otherwise, mean with single values in parentheses.

ity. Prior studies by Huang et al.,[22] using β-MeTrp in SRIF hexapeptide scaffold cyclo[Pro^6-Phe^7-DTrp^8-Lys^9-Thr^{10}-Phe^{11}], showed that the corresponding analogue with the (2*R*,3*S*)-β-MeTrp^8 (D-*threo*) substitution exhibited a "folded" conformation and significant changes in the receptor binding affinity by restricting the conformational freedom around the side chain. An IC_{50} < 1 nM for receptor binding (inhibition of ^{125}I-MK 678 on AtT-20 cells[23]) was reported. In that assay, the (2*R*,3*R*)-β-MeTrp^8- and the (2*S*,3*S*)-β-MeTrp^8-containing analogues showed extremely low binding affinity (IC_{50} > 1000 nM), while the (2*S*,3*R*)-β-MeTrp^8-containing analogue (L-*threo*) had an intermediate affinity (IC_{50} > 10 nM). Unfortunately, these results were published before the discovery of the sst's and selectivity could not be assessed. Upon these observations, we synthesized analogues **4**, **5**, **20**, and **23** containing only the threo isomers of β-MeTrp.

Substitution of Phe^7 in **2** and **3** by Tyr yielded **6** and **7**, which showed similar binding affinity profiles, namely that the introduction of DTrp^8 in this octapeptide scaffold tends to increase potency at all receptors, whereas the L-isomer tends to favor sst_4 selectivity. We concluded from these results that an L-amino acid at position 8 and an optimized substitution at position 7 (such as tyrosine, **6**) may lead to high sst_4 selectivity. In fact, H-c[Cys-Phe-Aph-Trp-Lys-Thr-Phe-Cys]-OH (**8**) and H-c[Cys-Phe-Aph-DTrp-Lys-Thr-Phe-Cys]-OH (**9**) have high sst_4 affinity (IC_{50} = 1.2 and 0.88 nM, respectively) and selectivity (>150-fold and 40-fold, respectively), suggesting that, indeed, an L-residue at position 8 will direct selectivity toward sst_4 and that concomitant substitutions at position 7 could improve selectivity. Unpredictably, H-c[Cys-Phe-Ala-Trp-Lys-Thr-Phe-Cys]-OH (**10**) and H-c[Cys-Phe-Ala-DTrp-Lys-Thr-Phe-Cys]-OH (**11**) have very high sst_4 affinity (IC_{50} = 0.84 and 0.98 nM, respectively) and selectivity (>600- and 200-fold, respectively), although, as mentioned earlier, there were recently published indications that [Ala^7]SRIF-NH_2 was indeed sst_4-selective, with moderate affinity.[14] Clearly, with such a simple analogue, we had achieved one of our stated goals of generating a potent, highly sst_4-selective ligand. Whether this analogue could be derivatized to yield an iodinatable tracer became our next priority. Because it is generally admitted that extensions at the N-terminus of SRIF and analogues are compatible with high potency, we made a series of tyrosinated analogues. The parent **12** also showed significant selectivity for sst_4 and high affinity for that receptor (IC_{50} = 1.6 nM). Additionally, we found that the combination of Tyr^2 and DTrp^8, such as in H-Tyr-c[Cys-Phe-Aph-DTrp-Lys-Thr-Phe-Cys]-OH (**14**) and H-Tyr-c[Cys-Phe-Ala-DTrp-Lys-Thr-Phe-Cys]-OH (**22**) or H-Tyr-c[Cys-Phe-Ala-D-*threo*-β-MeTrp-Lys-Thr-Phe-Cys]-OH (**23**), did not significantly affect the affinity of the analogue for sst_4 (IC_{50} = 1.2, 1.1, and 5.6 nM, respectively) but resulted in loss of selectivity, whereas the combination of Tyr^2 and LTrp^8, such as in H-Tyr-c[Cys-Phe-Aph-Trp-Lys-Thr-Phe-Cys]-OH (**13**) and H-Tyr-c[Cys-Phe-Ala-Trp-Lys-Thr-Phe-Cys]-OH (**19**), retained high affinity (IC_{50} = 1.9 and 1.98 nM, respectively) and sst_4 selectivity (>50 and >250, respectively). Unexpected was the observation that H-Tyr-c[Cys-Phe-Ala-L-*threo*-β-MeTrp-Lys-Thr-Phe-Cys]-OH (**20**) showed significant loss of selectivity (20-fold with respect to sst_2) yet retained high affinity for sst_4 (IC_{50} = 0.9 nM).

We had shown earlier that carbamoylation of the N-terminus of most SRIF analogues resulted in slightly improved affinity, selectivity, or both and confirmed these results with the synthesis of **15**−**17** and **24**. As expected, the three analogues with an LTrp^8 (**15**, **17**, and **24**) are highly potent and sst_4-selective, whereas the DTrp^8-containing analogue **16** is less selective than **15**.

Because of our need for radioiodinatable analogues, we synthesized the cold iodinated analogues of our most promising tracer precursors. In this case, cold monoiodination of **17** and **19** yielded **18** and **21**, with slightly decreased affinity for sst_4 and little effect to the binding affinity toward the other four receptors. We radiolabeled **19** with ^{125}I, purified the monoiodinated from the diiodinated analogues using HPLC, and tested its binding to sst_{1-5} cells. As seen in Figure 1, ^{125}I-**19** was able to strongly label sst_4 cells, whereas it did not bind to sst_{1-3} or sst_5 cells. Binding was detected both in sst_4-transfected CCL39 and HEK293 cells. In both cell types, binding of ^{125}I-**19** was found to be specific for somatostatin receptors of the sst_4 subtype, since the radioligand could be completely displaced not only by **10** but also by SRIF-28 and by the sst_4-selective non-peptide L-803,087 (Figure 2).

Because the Tyr^2 substitution did not negatively impact either affinity or selectivity, we investigated the effect of substitutions other than Tyr, Aph, or Ala at position 7 in the parent [Tyr^2]OLT-8 (**12**). Interestingly, substitutions by basic amino acids such as 4-aminomethylphenylalanine (Amp) (**25**, **26**), ornithine (**27**), or isopropyl-4-aminomethylphenylalanine (IAmp) (**29**) were well tolerated, but with a 2- to 3-fold loss of affinity and concomitant loss of selectivity. As in other pairs of analogues (**8**-**9**, **10**-**11**, **13**-**14**, and **15**-**16**), substitution of LTrp^8 with DTrp^8 in **29**-**30** resulted in increased affinity for sst_2 and sst_5, to the detriment of selectivity.

Tyrosine substitutions in SRIF and shorter analogues have traditionally been inserted at the N-terminus of the analogues (i.e., position 2 with deletion of residue 1) and at positions 7 and 11, where these were well tolerated, and not as a substitution of Phe^6, which was less favorable.[24] We therefore substituted Phe^{11} by Tyr^{11} in both OLT-8 and ODT-8 to yield **31** and **32**, respectively. In fact, this substitution was well tolerated in both analogues with the same tendency for sst_4 selectivity as had been shown for OLT-8 (**2**) and ODT-8 (**3**) themselves. Carbamoylation of **32** to yield **33**, however, led to some loss of affinity at sst_1 and sst_2. In this series, substitution of Phe^7 by Aph^7 led to dramatic changes in receptor selectivity, since **34** has retained the high affinity of its parent **31** with significant loss of affinity at sst_{1-3} and sst_5. Carbamoylation of **34** led to **35** with increased sst_4 selectivity and only limited loss of affinity.

It has not escaped our attention that analogues with >100- and 25-fold sst_4 selectivity and high affinity, such as **16** or **36**, respectively, with DTrp^8 and a blocked N-terminus could be more stable in a biological milieu than the highly sst_4-selective and potent **19**, for example.

Clearly, we have not synthesized and tested all possible combinations of substitutions at positions 2, 7,

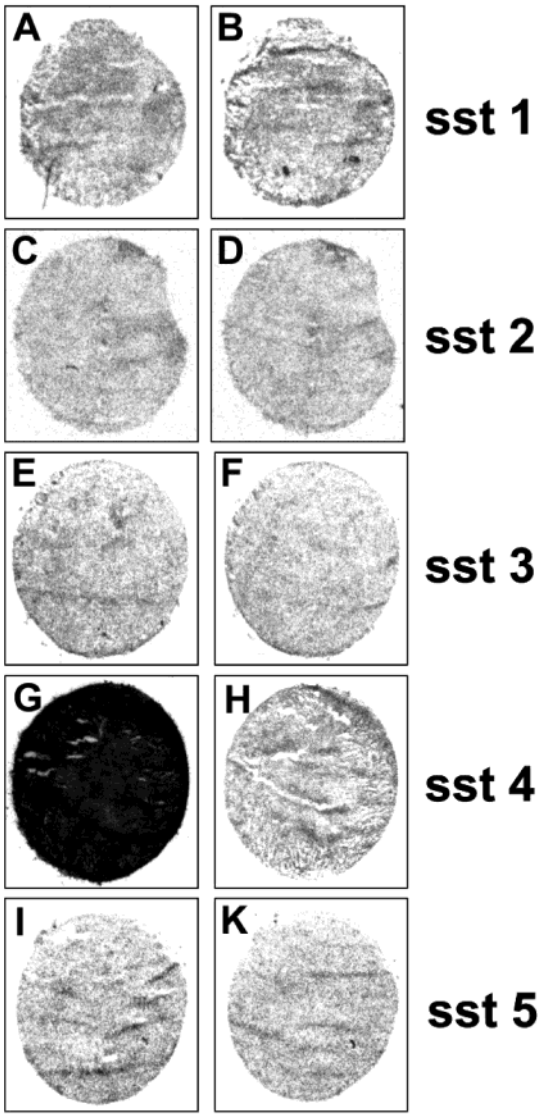

Figure 1. Binding of ^{125}I-**19** to sections containing cell pellets from sst$_1$-expressing CHO−K1 cells, sst$_2$-expressing CCL39 cells, sst$_3$-expressing CCL39 cells, sst$_4$-expressing CCL39 cells, and sst$_5$-expressing CHO−K1 cells. All sections were incubated with 30 000 cpm/100 μL of ligand. Strong specific labeling with the ligand is seen only in sst$_4$ cells (G). (A, C, E, G, I) Autoradiograms showing total binding of ^{125}I-**19** to sections of cell pellets expressing sst$_1$ (A), sst$_2$ (C), sst$_3$ (E), sst$_4$ (G), and sst$_5$ (I). (B, D, F, H, K) Autoradiograms showing nonspecific binding of ^{125}I-**19** (in the presence of **10** (10^{-6} M).

8, and 11 of OLT-8 and ODT-8 with and without carbamoylation of the N-terminus. However, several analogues in this series compared favorably with the non-peptide L-803,087 (**37**) in terms of affinity and selectivity. sst$_4$-selective analogues such as **8**−**11**, **16**, **17**, and **34** have more than twice the binding affinity of SRIF-28. Many others are equipotent to SRIF at sst$_4$. Finally, others may exhibit long duration of action in vivo once a clear function for sst$_4$ is assigned and an in vivo test is validated (see below).

To distinguish agonists from antagonists, the effect of the best sst$_4$-selective analogue on forskolin-stimulated cAMP production in sst$_4$-expressing HEK293 cells[25] was evaluated. Figure 3 illustrates the data. The agonist SRIF-28 potently inhibited forskolin-stimulated cAMP accumulation by more than 91% at a peptide

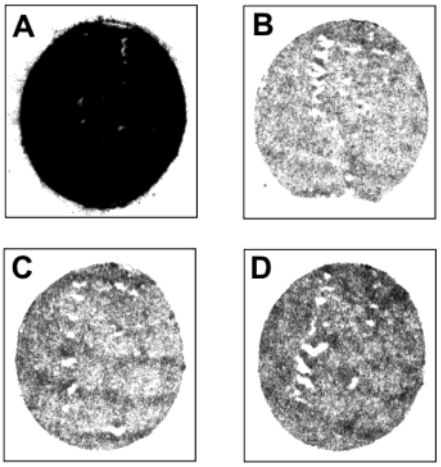

Figure 2. sst$_4$ specificity of the binding of ^{125}I-**19** in sections of sst$_4$-expressing CCL39 cell pellets. (A) Total binding of ^{125}I-**19**. (B) Binding in the presence of **10** (10^{-6} M). (C) Binding in the presence of SRIF-28 (**1**) (10^{-6} M). (D) Binding in the presence of L-803,087 (**37**) (10^{-6} M). Full displacement of ^{125}I-**19** is seen with all three compounds.

concentration of 100 nM, with an EC_{50} = 0.49 nM; it was used as control. Our best sst$_4$ analogue, **10**, was tested in this system and shown to have full agonistic properties similar to those of SRIF-28, with an EC_{50} = 1.12 nM. The sst$_3$-selective sst$_3$-ODN-8[11] was used as negative control in these experiments (Figure 3A). Analogues **8** and **21** are also agonists in this system, with EC_{50} values = 0.29 and 1.13 nM, respectively (Figure 3B). Analogue **8** was tested in this system because of its high affinity and selectivity and the fact that it differed significantly at position 7 from **10** (basic aromatic side chain versus the neutral aliphatic nature of the alanine side chain) and could therefore have been an antagonist. We tested **21** because it was going to be used as a tracer after radiolabeling and we wanted to confirm, indirectly, the agonistic activity of its parent **10**. Comparable results on cAMP production were obtained in sst$_4$-transfected CCL39 cells with these compounds (data not shown).

The present study describes a family of potent peptidic analogues of somatostatin with agonistic properties and high selectivity for the sst$_4$-receptor subtype. With its high sst$_4$ affinity, comparable to that of the natural SRIF-28, the best compound, **10**, is significantly better than any other peptidic sst$_4$-selective analogue and compares favorably with the recently discovered non-peptide agonist L-803,087 reported by Rohrer et al.[26] This series of novel analogues, having a 4-aminophenylalanine or even better, an alanine at position 7, combined with the native tryptophan residue at position 8 of the OLT-8 octapeptide scaffold, demonstrates that extraordinary sst$_4$ receptor selectivity can be achieved with rather simple substitutions. These findings however, leave open the challenge of identifying sst$_4$-selective antagonists.

The present study also permitted us to obtain a radioligand closely related to the structures of our best sst$_4$ compounds. This radioligand ^{125}I-**19** binds only to sst$_4$ cells and not to cells expressing the other four sst's, as shown by the complete displacement of the radioligand by SRIF-28 or by the sst$_4$-selective L-803,087. Such sst$_4$-selective agonist radioligands may be useful

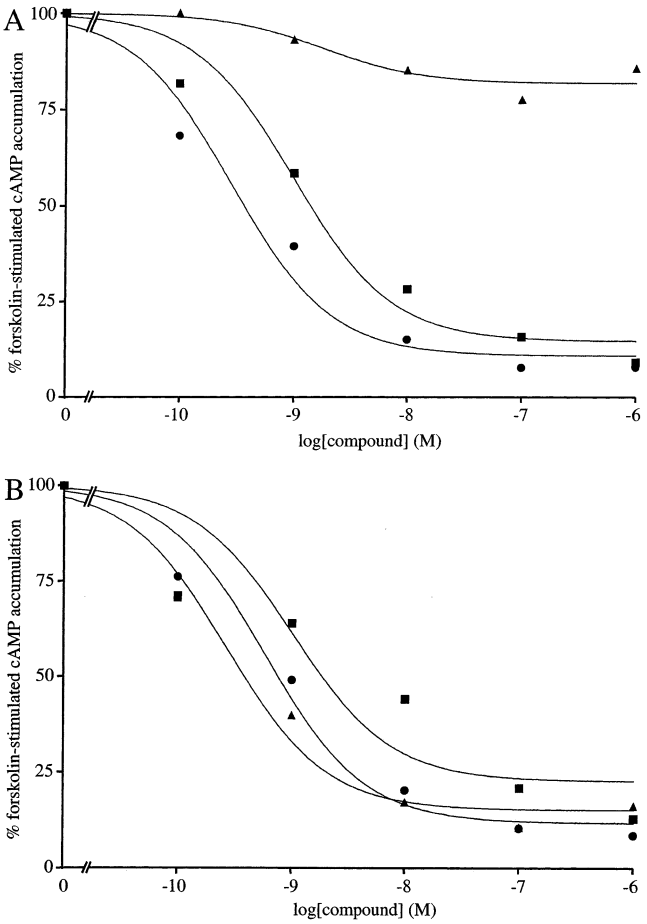

Figure 3. Effect of various concentrations of the sst_4-selective agonist **10** and the sst_3-selective antagonist sst_3-ODN-8 (A), as well as the sst_4-selective agonists **8** and **21** (B), in comparison with SRIF-28 as control, on forskolin-stimulated cAMP accumulation in HEK293 cells expressing sst_4. In (A) concentration−response curves were obtained with increasing concentrations of SRIF-28 (●) and **10** (■). sst_3-ODN-8 (▲), as negative control, has no effect. In (B) the effects of **8** (▲) and **21** (■) are shown in comparison to SRIF-28 (●). Data are expressed as % forskolin-stimulated cAMP accumulation. Each plot represents the mean of two independent experiments. Analogues **10**, **8,** and **21** behave as agonists.

in localizing sst_4 in human tissues and deciphering the functions mediated by sst_4. Presently, the sst_4 system is poorly understood, specifically in humans. Available information on sst_4 includes the observation that sst_4 is present in the rat brain, where somatostatin may play a neurotransmitter role,[27] and that sst_4 is also found in the rat lungs and in human placenta.[28,29]

It will also be worth evaluating the role of sst_4 in cancer. It is known that the sst_4 mRNA can be detected in some human tumors.[30−33] However, the incidence of the sst_4 protein appears to be extremely low[34] and its importance not well established in human tumors. Therefore, a confirmation of the presence or absence of the receptor protein, using sensitive receptor binding assays with selective radioligands such as ^{125}I-**19**, would be welcome. Those studies are underway.

The peptidic nature of our sst_4 agonists results in a number of advantageous properties: (a) their structures can be modified easily and rationally to obtain more potent or more stable compounds; (b) the relatively large molecules allow the addition of further isotopes, or even chelators with isotopes without significant modification of their binding affinity, to be used as clinical tools;[35,36] (c) a peptide will poorly cross the blood−brain barrier. If it is to be used for systemic application as a drug or radioligand in clinical indications involving peripheral targets, it will be a great advantage if it does not penetrate into the brain, an organ known to contain sst_4.[27]

To conclude, this study indicates that simple substitutions in the somatostatin molecule will yield potent sst_4-selective agonists that may be developed as tools for preclinical and clinical studies. An important sst_4-selective analogue (**19**) that can be easily radiolabeled has been identified. The fact that derivatives of the above retain high selectivity and affinity after carbamoylation of the N-terminus (**24** and **26**, for example) suggests that substitution of these analogues with chelating agents at the N-terminus may open the door to sst_4-selective radiotherapeutics (work in progress). Analogues such as **16** and **36** may have the additional advantage of being stable in a biological milieu. Finally, potent and sst_4-selective analogues selected from the three series described in this and the two preceding papers have unique conformations determined by NMR in dimethyl sulfoxide (a solvent that may mimic the receptor's environment), thus suggesting general structural parameters responsible for sst_4 selectivity.[37]

Experimental Section

Abbreviations. The abbreviations for the common amino acids are in accordance with the recommendations of the IUPAC−IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9−37). The symbols represent the L-isomer except when indicated otherwise. Additional abbreviations: Agl, aminoglycine; Amp, 4-aminomethylphenylalanine; Aph, 4-aminophenylalanine; *β*-MeTrp, *β*-methyltryptophan; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; Z(2Br), 2-bromobenzyloxycarbonyl; Z(2Cl), 2-chlorobenzyloxycarbonyl; Cbm, carbamoyl (H_2N-CO-); CZE, capillary zone electrophoresis; DIC, *N*,*N*′-diisopropylcarbodiimide; DIPEA, diisopropylethylamine; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; IAmp, isopropyl-4-aminomethylphenylalanine; (m-I)-Tyr, monoiodinated tyrosine; Mob, 4-methoxybenzyl; Nal, 3-(2-naphthyl)-alanine; *β*-Me2Nal, *β*-methyl-3-(2-naphthyl)alanine; NMP, *N*-methylpirrolidinone; OBzl, benzyl ester; SRIF, somatostatin-14; sst's, somatostatin receptors; TEA, triethylamine; TEAP, triethylammonium phosphate; TFA, trifluoroacetic acid.

Starting Materials. Boc-Cys(Mob)-CM resin, with a capacity of 0.3−0.4 mequiv/g, was prepared by the method reported by Horiki et al.[38] All Boc-N$^{\alpha}$-protected amino acids with side-chain protection {Cys(Mob), Lys[Z(2Cl)], Orn(Z), Thr(Bzl), Tyr-[Z(2Br)], and (m-I)-Tyr(3-BrBzl)} were commercially available (Bachem Inc., Torrance, CA; Chem Impex, Wood Dale, IL; Reanal, Budapest, Hungary), except for Boc-Aph(Fmoc), Boc-Amp(Z), and Boc-IAmp(Z),[39] which were synthesized in our laboratory. Additionally, the threo and erythro isomers of *β*-methyltryptophan were prepared according to the method reported by Snyder.[15] In short, indole (50 mmol) in glacial acetic acid (30 mL) was reacted with isopropylamine (56 mmol) and a solution of acetaldehyde (53 mmol) in benzene (10 mL) at 0 °C for 3 days to form 3-(isopropylaminoethylidene)indole (13.5 mmol, 27.1% yield). The 3-(isopropylaminoethylidene)-indole (11 mmol) was reacted with dibenzyl acetamidomalonate (11 mmol) and sodium methoxide (18.5 mmol) in toluene (15 mL) at 85 °C for 12 h, resulting in dibenzyl(3-indolylethylidene)acetamidomalonate (9 mmol, 90% yield), which was converted to ammonium (3-indolylethylidene)acetamidomalonate upon hydrogenolysis with 10% Pd/C catalyst (0.15 g) in

95% ethanol (25 mL) and addition of ammonium hydroxide (10 mL). The yield of this product was 96%. Decarboxylation and the formation of the unresolved 2-acetamido-3-(3-indolyl)-butyric acid (acetyl-β-methyltryptophan) was achieved by refluxing the ammonium (3-indolylethylidene)acetamidomalonate (6.4 mmol) in 6 mL of a pyridine:water (1:1) solvent mixture for 5 h. After dilution of the reaction mixture with water and acidification with 10% sulfuric acid to pH 3, the threo isomers were separated by fractional crystallization (45% yield) from the erythro isomers, which were obtained by ethyl acetate extractions of the mother liquor (41% yield). Hydrolysis of the separated acetyl threo and erythro isomers by refluxing with 4 N sulfuric acid for 5 h and neutralizing with saturated solution of $Ba(OH)_2$ resulted in the formation of the *threo*-(2*S*,3*R* and 2*R*,3*S*) and the *erythro*-(2*S*,3*S* and 2*R*,3*R*) isomers of 2-amino-3-(3-indolyl)butyric acid (β-methyltryptophan, β-MeTrp), with a yield of 64.5% and 54%, respectively. After preparative HPLC purification,[16] both diastereomers showed >95% purity, determined by CZE; the threo isomers migrated faster than the erythro isomers.[16,40] The threo isomers could be distinguished from the erythro isomers on the basis of the proton coupling constants between C_α−H and C_β−H, determined from ^{1}H NMR spectra. Diastereomers with larger coupling constant ($J_{H_\alpha-H_\beta} = 7.16$ Hz) were assigned the *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-MeTrp configuration, and diastereomers with smaller coupling constant ($J_{H_\alpha-H_\beta} = 4.41$ Hz) the *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-MeTrp configuration.[41,42] These isomers were converted to their N$^\alpha$-*tert*-butoxycarbonyl derivatives. For peptide synthesis, the unresolved N$^\alpha$-*tert*-butoxycarbonyl threo isomers were used to yield the epimeric peptide mixtures separated by RP-HPLC. The absolute configuration at the α-carbon of the *threo*-β-MeTrp in compounds **4**, **5**, **20**, and **23** (Table 1) was determined by enzymatic hydrolysis with trypsin, carboxypeptidase B, and carboxypeptidase A in three consecutive steps. Trypsin was able to quantitatively open the ring structure of compounds **4** and **20** within 2 h, whereas it took 2 days for partial opening of **5** and **23** after the lysine residue to yield the linear free acids, which eluted earlier on RP-HPLC and showed an 18-mass-unit increase. We further hydrolyzed the linear derivatives of **4** and **20** with carboxypeptidase B, which cleaved quantitatively the C-terminus lysine within 2 h, as shown by MS and a shift in RP-HPLC retention time toward the more hydrophobic region. Finally, these products were completely digested with carboxypeptidase A in 20 min, suggesting that all amino acids in the remaining sequence were of the L-configuration. One of the free amino acids (β-MeTrp) could be identified as one of the hydrolysis product by RP-HPLC. This procedure is described in more detail in the preceding papers.[12,13]

All reagents and solvents were ACS grade and were used without further purification.

Peptide Synthesis. Peptides were synthesized by the solid-phase approach, either manually or on a CS-Bio peptide synthesizer, model CS536.[43] Peptide couplings were mediated for 1 h by DIC in CH_2Cl_2 or NMP and monitored by the qualitative ninhydrin test. A 3-equiv excess of amino acid based on the original substitution of the resin was used in most cases. Boc removal was achieved with trifluoroacetic acid (60% in CH_2Cl_2, 1−2% *m*-cresol) for 20 min. An isopropyl alcohol (1% *m*-cresol) wash followed TFA treatment, and then successive washes with triethylamine solution (10% in CH_2Cl_2), methanol, triethylamine solution, methanol, and CH_2Cl_2 completed the neutralization sequence. The ureido group (Cbm) at the N-terminus of **15**−**18**, **24**, **26**−**30**, **33**, **35**, **36** was introduced on the resin. The N-terminal Boc group of the fully assembled peptide was deprotected with TFA in the usual manner,[10,18] and after neutralization, the carbamoylation proceeded with NaOCN (100 mg, 0.65 mmol) in NMP (4 mL) and glacial acetic acid (3 mL per gram of initial resin). The mixture was agitated at room temperature for 30 min, and ninhydrin test indicated a complete reaction. The completed peptide was then cleaved from the resin by HF containing the scavengers anisole (10% v/v) and methyl sulfide (5% v/v) for 60 min at 0 °C. The diethyl ether-precipitated crude peptides were cyclized in 75% acetic acid (200 mL) by addition of iodine (10% solution in methanol) until the appearance of a stable orange color. Forty minutes later, ascorbic acid was added to quench the excess of iodine.

Purification of Peptides. The crude, lyophilized peptides were purified by preparative RP-HPLC[16] on a 5-cm × 30-cm cartridge, packed in the laboratory with reversed-phase 300 Å Vydac C_{18} silica (15−20 μm particle size) using a Waters Associates Prep LC/System 500A system. The peptides eluted with a flow rate of 100 mL/min, using a linear gradient of 1% B per 3 min increase from the baseline % B (eluent A = 0.25 N TEAP, pH 2.25; eluent B = 60% CH_3CN, 40% A). All peptides were subjected to a second purification step, carried out with eluents A = 0.1% TFA in water and B = 60% CH_3CN/40% A on the same cartridge using a linear gradient of 1% B per min increase from the baseline % B. Analytical HPLC screening of the purification was performed on a Vydac C_{18} column (0.46 × 25 cm, 5 μm particle size, 300 Å pore size) connected to a Rheodyne injector, two Beckman 100A pumps, a model 420 system controller programmer, a Kratos 750 UV detector, and a Houston Instruments D-5000 strip chart recorder. The fractions containing the product were pooled and subjected to lyophilization.

Characterization of SRIF Analogues. (See Table 1 footnote.) The purity of the final peptides was determined by analytical RP-HPLC, performed with a linear gradient using 0.1 M TEAP (pH 2.5) as eluent A and 60% CH_3CN/40% A as eluent B on a Hewlett-Packard Series II 1090 liquid chromatograph connected to a Vydac C_{18} column (0.21 × 15 cm, 5 μm particle size, 300 Å pore size), controller model 362, and a Think Jet printer. CZE analysis was performed as described earlier.[17] Each peptide was found to have a purity of >90% by HPLC and CZE. Mass spectra (MALDI-MS) were measured on an ABI-Perseptive DE-STR instrument. The instrument employs a nitrogen laser (337 nm) at a repetition rate of 20 Hz. The applied accelerating voltage was 20 kV. Spectra were recorded in delayed extraction mode (300 ns delay). All spectra were recorded in the positive reflector mode. Spectra were sums of 100 laser shots. Matrix α-cyano-4-hydroxycinnamic acid was prepared as saturated solutions in 0.3% trifluoroacetic acid and 50% acetonitrile. The observed monoisotopic $(M + H)^+$ values of each peptide corresponded with the calculated $(M + H)^+$ values.

Receptor Autoradiography. CHO-K1, CCL39, and HEK293 cells stably expressing the cloned five human sst's were grown as described previously.[18,25] All cell culture reagents were supplied by Gibco BRL, Life Technologies (Grand Island, NY). The receptor autoradiographical experiments were performed as reported earlier.[18]

Adenylate Cyclase Activity. Modulation of forskolin-stimulated adenylate cyclase activity was determined using a radioimmunoassay measuring intracellular cAMP levels by competition binding. sst$_4$-expressing HEK293 and CCL39 cells[11,25,44] were subcultured in poly-D-lysine-coated 96-well culture plates at 2×10^4 cells/well and grown for 24 h. Culture medium was removed from the wells, and 100 μL of fresh medium containing 0.5 mM 3-isobutyl-I-methylxanthine (IBMX) was added to each well. Cells were incubated for 30 min at 37 °C. Medium was then removed and replaced with fresh medium containing 0.5 mM IBMX, with or without 10 μM forskolin and various concentrations of peptides. Cells were incubated for 30 min at 37 °C. After removal of medium, cells were lysed, and cAMP accumulation was determined using a commercially available cAMP scintillation proximity assay (SPA) system according to the instructions from the manufacturer (RPA 538, Amersham Biosciences, Little Chalfont, UK). cAMP data were expressed as percentage of stimulation over the nonstimulated level. Values of EC_{50} (the agonist concentration causing 50% of its maximal effect) were derived from concentration−response curves.

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