Novel sst_4-Selective Somatostatin (SRIF) Agonists. 2. Analogues with β-Methyl-3-(2-naphthyl)alanine Substitutions at Position 8[⊥]

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We present a family of human sst_4-selective, high-affinity (IC_{50} = 2−4 nM) cyclic somatostatin (SRIF) octapeptides. These peptides result from the substitution of DTrp[8] in H-c[Cys[3]-Phe[6]-Phe[7]-DTrp[8]-Lys[9]-Thr[10]-Phe[11]-Cys[14]]-OH (SRIF numbering) (ODT-8) by one of the four conformationally biased stereoisomers of β-methyl-3-(2-naphthyl)alanine (β-Me2Nal). Whereas H-c[Cys-Phe-Phe-DNal-Lys-Thr-Phe-Cys]-OH (ODN-8, **2**) has high affinity and marginal selectivity for human sst_3 (Reubi et al., *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13973−13978), H-c[Cys-Phe-Tyr-D-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH (**5**) has high affinity for all sst's except for sst_1; H-c[Cys-Phe-Tyr-L-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH (**6**) has high affinity for sst_4 (IC_{50} = 2.1 nM), with more than 50-fold selectivity toward the other receptors. Analogues **7** and **8**, containing D- and L-*erythro*-β-Me2Nal instead of the corresponding threo derivatives at position 8, are essentially inactive at all receptors. Substitution of Tyr[7] in **5** and **6** by Aph[7] resulted in **9** and **10** with similar affinity patterns overall yet lowered affinity. The substitution of DCys[3] for Cys[3] in **5** and **6** yielded H-c[DCys-Phe-Tyr-D-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH (**11**) and H-c[DCys-Phe-Tyr-L-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH (**12**), with biological profiles almost identical to those of their parents **5** and **6** (i.e., high affinity for sst_{2-5} for **11** and high affinity and selectivity for sst_4 for **12**). Analogue **12**, with high sst_4 affinity combined with the highest sst_4 selectivity among all tested compounds, is an agonist in the cAMP accumulation assay (EC_{50} = 1.29 nM). Cold monoiodination of **12** yielded **14**, with loss of sst_4 selectivity and loss of high affinity (IC_{50} = 21 nM). Introduction of Tyr[2] in **9** and **10** and substitution of Cys[3] by DCys[3], to yield **15** and **16** (IC_{50} = 9.8 and 61 nM, respectively, for sst_4 and limited selectivity), failed to generate a high-affinity ^{125}iodinatable sst_4-selective ligand. Substitution of Phe by Tyr at position 11 in H-c[DCys-Phe-Phe-L-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH yielded **18** (IC_{50} = 11.8 nM at sst_4), with limited sst_4 selectivity (30-fold or greater at the other receptors) yet only slightly improved affinity over that of **14**. Cold monoiodination of **18** yielded **20** (IC_{50} = 30 nM at sst_4 and high selectivity). Whereas we were able, in this study, to identify a new family of sst_4-selective, high-affinity compounds, our additional goal, to identify highly potent and sst_4-selective ligands amenable to ^{125}iodination, could not be achieved satisfactorily. On the other hand, some of the diastereomers identified in this study, such as **5**, **11**, **17**, and **19**, are very potent ligands at all receptors but sst_1.

Introduction

SRIF (H-Ala[1]-Gly[2]-c[Cys[3]-Lys[4]-Asn[5]-Phe[6]-Phe[7]-Trp[8]-Lys[9]-Thr[10]-Phe[11]-Thr[12]-Ser[13]-Cys[14]]-OH) is a major endocrine hormone with multiple physiological actions.[1−11] The actions of SRIF are modulated by one or more of the five known membrane-associated receptor subtypes: sst_1, sst_2, sst_3, sst_4, and sst_5.[12,13] The biological role as well as the cellular distribution of each receptor subtype is far from being completely understood. Very little is known about functions associated with sst_4 and its localization in normal and diseased human tissues and tumors. There are some speculations that sst_4 may act as a counter-regulatory receptor.[14] It is also presumed that this receptor may be important in the treatment of glaucoma-related diseases.[15] sst_4 receptors are present in rat lung sections,[16] human placenta,[17] human central nervous system,[18] rat brain,[19] including many rat forebrain regions,[20] and human ovarian tumors,[21] but it is not found in significant abundance in normal human tissues as are some of the other receptors. Over the past three decades, hundreds of SRIF analogues were reported and tested for their affinity and selectivity toward the five receptor subtypes (sst's).[10] None of the three commercially available SRIF-based drugs, characterized by their high affinity for sst_2, lantreotide, vapreotide, or octreotide, has a particularly high affinity for the human sst_4 receptor.[12,22,23] Reports

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of sst_4-selective ligands include non-peptide ligands,[24,25,26] a linear small β-peptide which can fold to a turn structure,[27] a lactam backbone cyclic SRIF analogue with high affinity for sst_4, but without selectivity,[28] and an SRIF analogue with modest sst_4 affinity, limited selectivity, and lacking the commonly found type II′ β-turn.[29] Except for the non-peptide L-803,087,[25] high-affinity sst_4 ligands, selective by a factor >100, have not been described. Because peptide ligands would have different pharmacokinetics than non-peptides, both peripherally and centrally, it was of great interest to us to design potent sst_4-selective peptides for sst_4 localization and determination of its physiological function.

It has been demonstrated that the side-chain conformation of an amino acid in a peptide can be controlled by introducing an alkyl/aryl group at the β-position. This modification does not perturb the backbone conformation significantly and still allows the peptide backbone and side chain some degree of flexibility, which often is necessary and even crucial for peptide activity.[30] The incorporation of amino acids capable of restricting conformational freedom around the side chain in χ space is a powerful approach in peptide ligand design.[31] Replacement of either the *pro-R* or *pro-S* β-hydrogens by a methyl group in amino acids increases their size and their lipophilicity and causes one of the allowable conformations to be significantly more stable than the other due to steric interactions.[32] In the past 15 years, several conformationally constrained β-methyl-substituted amino acids, such as β-methylphenylalanine,[33−36] β-methyltyrosine,[37] β-methyl-2′,6′-dimethyltyrosine,[32] β-methyltryptophan,[31,38−42] β-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid,[43] and β-methylnaphthylalanine,[44] were synthesized and introduced into bioactive peptides, causing large differences in receptor binding and selectivity. In some cases, such substitutions also prolonged the biological activity of the given peptides by locking a favorable "bioactive conformation".[32,45−53] We have discussed in the preceding paper[54] and others the effect of β-methylation of betidamino acids on their Ramachandran plots (structural studies)[55] and of betidamino acid-containing bioactive peptides on their biological activities.[56] These findings suggest that β-alkylation is a powerful approach in peptide ligand design and has great potential for understanding ligand−receptor interactions. In the field of SRIF, Huang et al.[45] methylated the side-chain β-carbon of Trp^8 in SRIF hexapeptide cyclo[Pro^6-Phe^7-DTrp^8-Lys^9-Thr^{10}-Phe^{11}]. In this scaffold, considerable conformational flexibility around the backbones of Phe^7 and Thr^{10} and the side chains of Phe^7, Trp^8, and Phe^{11} was observed. By the introduction of α- and β-methylated amino acids into this scaffold, it was observed that the analogue with (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[57]) 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 affinity (IC_{50} > 1000 nM), while the (2*S*,3*R*)-β-$MeTrp^8$ 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. Whereas we had shown that significantly shortened analogues of SRIF, such as H-c[Cys^3-Phe^6-Phe^7-DTrp^8-Lys^9-Thr^{10}-Phe^{11}-Cys^{14}]-OH (des-$AA^{1,2,4,5,12,13}$-[DTrp^8]SRIF; ODT-8), retained high potency for the different biological activities testable at the time,[58−60] we were able to confirm more recently that these analogues in fact retained high affinity for all sst's, with the possible exception of sst_1.[56] In these studies, we showed that the substitution of DAgl(N$^{\beta}$Me,2naphthoyl) (the betide homologue of β-methyl-DNal) for DTrp at position 8, Tyr for Phe at position 7, and carbamoylation at the N-terminus in this scaffold led to the highly potent (IC_{50} = 6.7 nM) sst_3-selective (with more than 1000-fold selectivity toward all other sst's) H_2N-CO-c[DCys-Phe-Tyr-DAgl(N$^{\beta}$Me,-2naphthoyl)-Lys-Thr-Phe-Cys]-OH (des-$AA^{1,2,4,5,12,13}$-[$DCys^3$,Tyr^7,DAgl(N$^{\beta}$Me,2naphthoyl^8]Cbm-SRIF; sst_3-ODN-8).[56]

Herein, we report a study to further enhance the potency and selectivity of our scaffold by introducing the conformationally constrained optical isomers of β-methyl-3-(2-naphthyl)alanine (β-Me2Nal) at position 8 of H-c[Cys^3-Phe^6-Phe^7-DNal^8-Lys^9-Thr^{10}-Phe^{11}-Cys^{14}]-OH (ODN-8), leading to analogues with high affinity and selectivity for human sst_4. The synthesis and separation of the isomers of β-Me2Nal are described in detail. The binding affinities of the analogues were derived from competition experiments on cells transfected with the five human SRIF receptor subtypes.[56] The most potent and selective analogues were evaluated for their agonist/antagonist properties, compliant with the modulation of forskolin-stimulated adenylate cyclase activity.

Results and Discussion

The literature offers two methods for the synthesis of β-Me2Nal. Whereas the stereoselective method of Yuan et al. provides each of the four stereoisomers,[44] the method of Kataoka et al.[33] is nonstereoselective and provides a mixture of the four stereoisomers in unequal amounts (55% erythro and 45% threo, determined by HPLC). We chose and modified the latter method because it may be simpler and more economical to scale-up than the stereoselective approach. We describe this method in Scheme 1. In short, 2-(1-bromoethyl)naphthalene was prepared by the addition of hydrogen bromide to 2-vinylnaphthalene. Condensation with diethyl acetamidomalonate (reacted with sodium ethylate) yielded the 2-acetylamino-2-(1-naphthalen-2-ylethyl)-malonic acid diethyl ester, which was hydrolyzed and decarboxylated by refluxing in the presence of 6 N HCl/glacial acetic acid (1/1) without the isolation of the intermediate product. In Scheme 2, we describe the separation of the *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-Me2Nal·HCl from the *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-Me2Nal·HCl diastereomers by fractional crystallization. The less soluble erythro enantiomers of β-Me2Nal crystallized first from the water:ethanol (1:2) solvent mixture. After the pH of the mother liquor was adjusted to 6.5 with ammonium hydroxide, the threo enantiomers precipitated. Several recrystallizations from 1:1 mixtures of water and ethanol of both substances afforded the diastereomers with an optical purity >95%. The configurations of the diastereomers were determined indirectly by the measurement of the proton coupling

Scheme 1. Synthetic Route of β-Methyl-3-(2-naphthyl)alanine

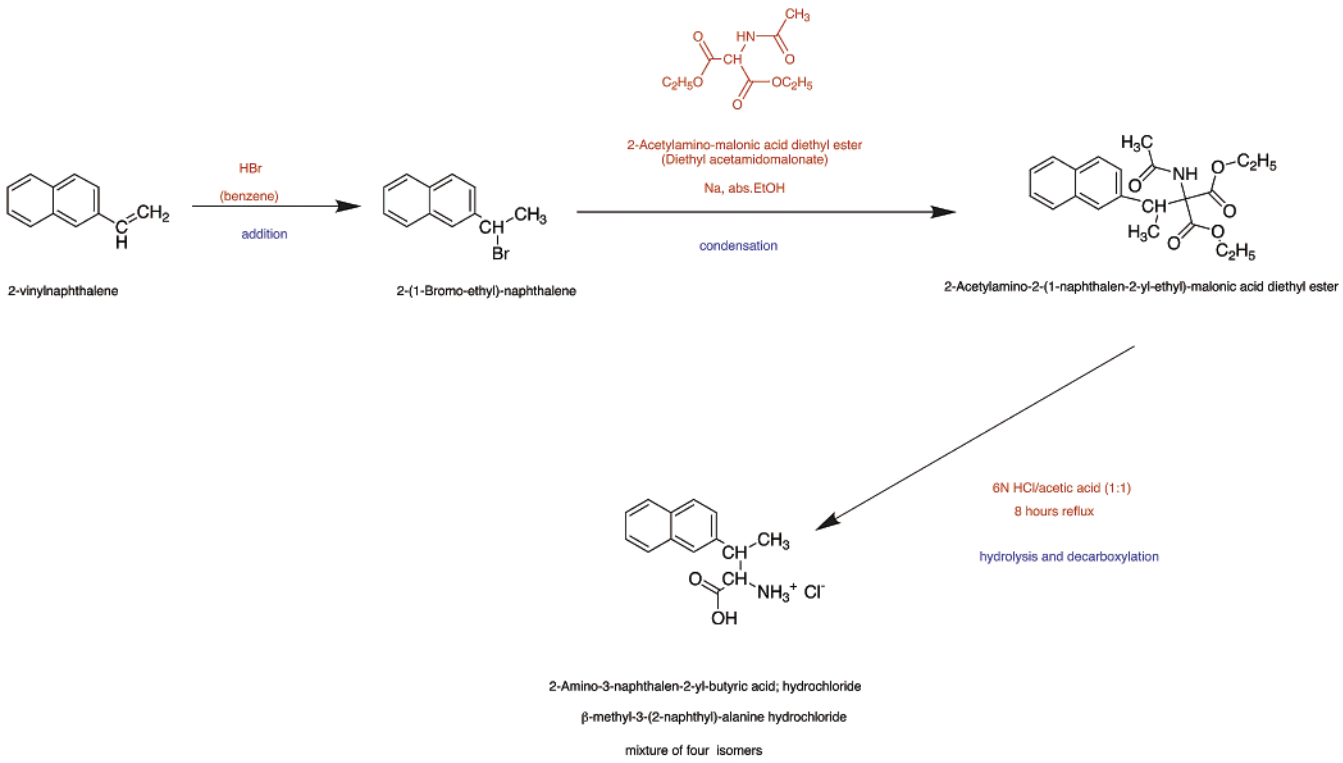


Table 1. Physicochemical Properties of sst$_4$-Selective Analogues and Control Peptides

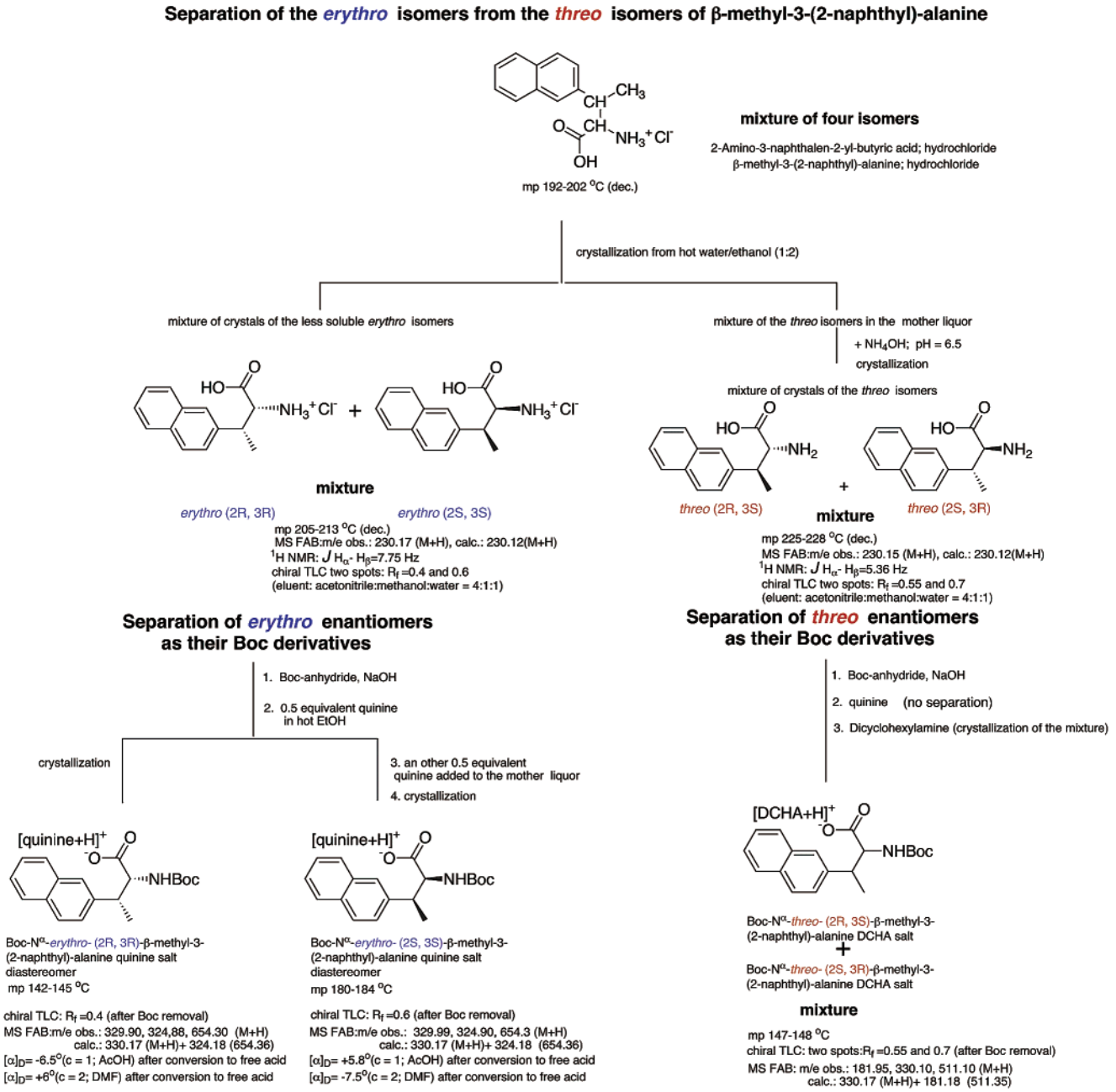
		purity (%)		MS[c]	
no.	compd	HPLC[a]	CZE[b]	M (mono) calcd	MH$^+$ (mono) obsd
1	Somatostatin-28, SRIF-28	98	98	3146.5	3147.7
2	H-c[Cys-Phe-Phe-D-Nal-Lys-Thr-Phe-Cys]-OH (ODN-8)	99	98	1089.46	1090.5
3	H-c[Cys-Phe-Tyr-D-Nal-Lys-Thr-Phe-Cys]-OH	99	99	1105.44	1106.4
4	H-c[D-Cys-Phe-Tyr-D-Nal-Lys-Thr-Phe-Cys]-OH	99	98	1105.44	1106.3
5	H-c[Cys-Phe-Tyr-D-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH	95	99	1119.46	1120.3
6	H-c[Cys-Phe-Tyr-L-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH	99	99	1119.46	1120.4
7	H-c[Cys-Phe-Tyr-D-*erythro*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH	99	95	1119.46	1120.4
8	H-c[Cys-Phe-Tyr-L-*erythro*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH	99	99	1119.46	1120.4
9	H-c[Cys-Phe-Aph-D-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH	95	99	1118.47	1119.5
10	H-c[Cys-Phe-Aph-L-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH	98	99	1118.47	1119.5
11	H-c[D-Cys-Phe-Tyr-D-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH	99	97	1119.46	1120.4
12	H-c[D-Cys-Phe-Tyr-L-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH	99	97	1119.46	1120.4
13	H-c[D-Cys-Phe-(m-I)-Tyr-D-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH	97	98	1245.35	1246.3
14	H-c[D-Cys-Phe-(m-I)-Tyr-L-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH	98	98	1245.35	1246.4
15	H-Tyr-c[D-Cys-Phe-Aph-D-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH	97	89	1324.55	1325.5
16	H-Tyr-c[D-Cys-Phe-Aph-L-*threo*-β-Me2Nal-Lys-Thr-Phe-Cys]-OH	92	94	1324.55	1325.5
17	H-c[D-Cys-Phe-Phe-D-*threo*-β-Me2Nal-Lys-Thr-Tyr-Cys]-OH	99	98	1119.39	1120.5
18	H-c[D-Cys-Phe-Phe-L-*threo*-β-Me2Nal-Lys-Thr-Tyr-Cys]-OH	99	98	1119.39	1120.6
19	H-c[D-Cys-Phe-Phe-D-*threo*-β-Me2Nal-Lys-Thr-(m-I)-Tyr-Cys]-OH	99	98	1245.35	1246.4
20	H-c[D-Cys-Phe-Phe-L-*threo*-β-Me2Nal-Lys-Thr-(m-I)-Tyr-Cys]-OH	99	98	1245.35	1246.4
21	L-803,087 (Merck)[25]				

[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.

constants between C_α−H and C_β−H, determined from their ^{1}H NMR spectra.[33,61] The purity of the diastereomers was determined by capillary zone electrophoresis (CZE).[62] The erythro enantiomers were resolved as the quinine salts of the Boc derivatives. The threo enantiomers could not be separated by crystallization of their quinine salts. However, the peptides in which these enantiomers were introduced could be easily separated by HPLC, and the final configuration of the α-carbon was determined by enzymatic hydrolysis of the L-isomers and resistance to degradation of the peptides containing the D-isomers (see Experimental Section).

All analogues shown in Table 1 were synthesized either manually or automatically on a chloromethylated resin using the Boc strategy. The peptides were purified using preparative RP-HPLC in at least two different solvent systems (TEAP, pH 2.25, and 0.1% TFA on C_{18} silica) and characterized as shown in Table 1 using RP-

Scheme 2. Separation of the Four Isomers of β-Methyl-3-(2-naphthyl)alanine



HPLC, CZE, and mass spectrometry as published earlier.[63] The compounds were tested for their ability to bind to the five human sst receptor subtypes in competitive experiments using ^{125}I-[Leu^8,DTrp^{22},Tyr^{25}]-SRIF-28 as radioligand (Table 2). Cells stably expressing the cloned five human sst's were grown as described previously.[64] Cell membrane pellets were prepared and receptor autoradiography was performed as depicted in detail previously.[64] The binding affinities are expressed as IC_{50} values that were calculated after quantification of the data using a computer-assisted image-processing system as described previously.[65,64] One of the most potent analogues (**12**) was evaluated for its agonist/antagonist properties, measuring inhibition of cyclic adenosine monophosphate (cAMP) production. Control peptides include SRIF-28, H-c[Cys-Phe-Phe-DNal-Lys-Thr-Phe-Cys]-OH (ODN-8) (**2**), and the sst_4-selective non-peptide L-803,087 (**21**).[25]

We previously published that the introduction of DNal at position 8 into our cyclo[Cys^3-Phe^6-Phe^7-DTrp^8-Lys^9-Thr^{10}-Phe^{11}-Cys^{14}] scaffold (ODT-8) resulted in ODN-8 (**2**) octapeptide, which showed a tendency toward sst_3 selectivity as compared to ODT-8, which exhibited a tendency for sst_4 selectivity. From this lead, we developed analogues with high binding affinity and selectivity for the human sst_3 receptor subtype.[56] In short, we showed that chiral inversion at position 3, the substitution of DAgl(N$^{\beta}$Me,2naphthoyl) for DNal at position 8, and carbamoylation at the N-terminus led to the analogue H_2N-CO-c[DCys-Phe-Tyr-DAgl(N$^{\beta}$Me,2naphthoyl-Lys-Thr-Phe-Cys]-OH (sst_3-ODN-8), which showed binding affinity equal to that of SRIF-28 for sst_3 and less than one-thousandth of that for the other four SRIF receptor subtypes.[56] We hypothesized that the ring size of the analogue and the unique conformational character of the *N*-methylated amino-2-naphthoyl side chain

Table 2. sst$_{1-5}$ Binding Affinities (IC$_{50}$, nM) of sst$_4$-Selective Analogues and Control Peptides

	IC$_{50}$ (nM)[a]				
no.	sst$_1$	sst$_2$	sst$_3$	sst$_4$	sst$_5$
1	3.8 ± 0.4 (25)	2.7 ± 0.2 (25)	4.9 ± 0.5 (24)	3.1 ± 0.2 (24)	2.9 ± 0.2 (24)
2	607 ± 168 (3)	173 ± 41 (3)	6.7 ± 1.9 (3)	41 ± 19 (3)	34 ± 10 (3)
3	>1000 (2)	57 (63; 51)	3.4 (3.2; 3.5)	1.4 (1.6; 1.1)	13 (14; 13)
4	>1000 (2)	24 (25; 23)	3.1 (2.9; 3.2)	1.2 (0.95; 1.5)	9 (9.5; 8.4)
5	410 (300; 520)	30 (30; 30)	18 (22; 14)	2.2 (4; 0.55)	17.5 (18; 17)
6	>1000 (4)	110 ± 15 (3)	600 ± 255 (3)	2.1 ± 0.6 (4)	147 ± 3.3 (4)
7	>1000 (2)	>1000 (2)	>10K (2)	105 (160; 50)	>1000 (2)
8	>10K (1)	>10K (1)	>1000 (1)	>1000 (1)	>10K (1)
9	>1000 (2)	102 (79; 125)	186 (130; 241)	8.7 (8; 9.4)	101 (70; 131)
10	>10K (2)	575 (400; 750)	>1000 (2)	6.9 (6.5; 7.2)	>1000 (2)
11	545 ± 122 (4)	12 ± 2 (4)	14 ± 3 (4)	0.53 ± 0.04 (3)	27 ± 5.6 (3)
12	>10K (6)	339 ± 103 (5)	664 ± 81 (5)	3.5 ± 0.5 (6)	668 ± 86 (6)
13	>1000 (3)	22 ± 16 (3)	61 ± 47 (3)	12 ± 8.6 (3)	152 ± 93 (3)
14	>10K (3)	673 ± 368 (3)	697 ± 118 (3)	21 ± 2.7 (3)	>1000 (3)
15	>1000 (2)	204 (150; 258)	171 (98; 244)	9.8 (8; 11.5)	127 (170; 83)
16	>1000 (2)	474 (420; 527)	595 (340; 850)	61 (42; 79)	>1000 (2)
17	>1000 (3)	16 ± 11 (3)	12 ± 6 (3)	9.2 ± 5 (3)	47 ± 20 (3)
18	>1000 (4)	357 ± 131 (3)	325 ± 84 (4)	11.8 ± 3 (3)	700 ± 200 (3)
19	778 ± 109 (3)	26 ± 7.4 (3)	9.7 ± 2.2 (3)	1.8 ± 0.3 (3)	23 ± 16 (3)
20	>10K (4)	>1000 (3)	>1000 (3)	30 ± 5.1 (4)	>1000 (3)
21	>1000 (4)	>10K (4)	>10K (4)	4.2 ± 1.9 (3)	>10K (4)

[a] The IC$_{50}$ values (nM) were derived from competitive radioligand displacement assays reflect the affinities of the analogues for the cloned human somatostatin receptors using the nonselective ^{125}I-[Leu^8,DTrp^{22},Tyr^{25}]SRIF-28 as the radioligand. Mean value ± SEM when $N \geq 3$ (shown in parentheses). In other cases, values are listed in parentheses.

of Agl were critical for the sst$_3$ selectivity and high affinity. When we compared the affinities of **2** versus **3**, we found that substitution of the Tyr residue for Phe at position 7, to generate peptides suitable for radioiodination, had no significant effect on the binding affinity for sst$_1$, sst$_2$, sst$_3$, or sst$_5$, but a 20-fold increase in affinity for sst$_4$. Additionally, the substitution of DCys in **4** for Cys at position 3 in **3** did not affect the profile of binding properties of these analogues (Table 2).

To further explore the structural role of positions 7 and 8 in the ODT-8/ODN-8 scaffold, we have prepared the four isomers of *β*-Me2Nal, the closely related homologue of the betidamino acid Agl(N$^{\beta}$Me,2naphthoyl[8] found in sst$_3$-ODN-8,[56] and substituted them for DNal in **3** (see analogues **5**−**8** in Table 1). This substitution greatly modified the biological profile (binding affinity and selectivity for sst$_4$) of these analogues (Table 2). Whereas **5** has high affinity for all sst's except sst$_1$, the insertion of the L-threo isomer of *β*-Me2Nal in **6** (versus the D-threo isomer in **5**) was accompanied by marked reduction in binding affinity for all receptor subtypes, except for sst$_4$ relative to **5**. H-c[Cys-Phe-Tyr-L-*threo*-*β*-Me2Nal-Lys-Thr-Phe-Cys]-OH (**6**) binds to sst$_4$ with an IC$_{50}$ of 2.1 ± 0.6 nM, and it is highly selective for sst$_4$ over sst$_{1-3}$ and sst$_5$ by factors of 500, 50, 200, and 70, respectively. Binding affinity, on the other hand, was completely lost at all receptors in **7** and **8**, which indicates that the threo configuration is favored over the erythro configuration. Our findings are in agreement with the results of Huang et al., who found that only the (2*R*,3*S*)- and (2*S*,3*R*)-*β*-MeTrp isomers were allowed at position 8 in the potent c[Pro^6-Phe^7-DTrp^8-Lys^9-Thr^{10}-Phe^{11}] SRIF analogue.[45]

Because **3** was altogether a more potent analogue than **2**, where the only difference is a Tyr at position 7 for a Phe^7 in **2**, position 7 was further explored by the introduction of a 4-aminophenylalanine (Aph) in **5** and **6**, to yield H-c[Cys-Phe-Aph-D-*threo*-*β*-Me2Nal-Lys-Thr-Phe-Cys]-OH (**9**) and H-c[Cys-Phe-Aph-L-*threo*-*β*-Me2Nal-Lys-Thr-Phe-Cys]-OH (**10**). The profiles of affinities of the pairs **5**, **9** and **6**, **10** are very similar, although the substitution seems to be slightly less favorable. Again, the L-*threo*-*β*-Me2Nal^8-containing **10** showed more selectivity for sst$_4$, with an IC$_{50}$ of 6.9 nM, and practically no binding affinity for the other four receptors, other than the D-*threo*-*β*-Me2Nal^8-containing **9**, with an IC$_{50}$ of 8.7 nM for sst$_4$ and almost equal binding affinities (100−200 nM) for all other receptors except sst$_1$ (>1000 nM).

On the basis of literature precedence in octreotide-type analogues, the D-AA^2-LCys^3-containing SRIF analogues function as agonists, whereas the L-AA^2-DCys^3-substituted homologues function as antagonists.[66,67,68] Our sst$_3$-ODN-8 analogue supported the application of this paradigm.[56] In this new series, we have synthesized several analogues containing DCys substitution in position 3 with the same intention (**4** and **11**−**20**). Although, at first sight, this substitution does not seem to affect the selective binding of these peptides relative to the parent LCys^3-containing analogues, H-c[DCys-Phe-Tyr-L-*threo*-*β*-Me2Nal-Lys-Thr-Phe-Lys]-OH (**12**) is slightly more sst$_4$-selective than **6** (>10K nM versus >1000 nM at sst$_1$, 339 nM versus 110 nM at sst$_2$, 664 nM versus 600 nM at sst$_3$, and 668 nM versus 147 nM at sst$_5$), with equally high affinity for sst$_4$ (IC$_{50}$ = 3.5 ± 0.5 and 2.1 ± 0.6 nM, respectively). Tested in the cAMP accumulation assay with CCL39 cells, **12** inhibited forskolin-stimulated cAMP accumulation by more than 50% at a peptide concentration of 1 nM and is therefore an agonist (Figure 1). Its EC$_{50}$ value is 1.29 ± 1.17 nM, compared to 0.19 ± 0.06 nM for SRIF-28. Compound **6** is also an agonist in the cAMP accumulation assay (data not shown).

We then synthesized the cold iodinated analogues of our most promising tracer precursors to fulfill our pressing need for a radioligand. In this case, substitution of Tyr by monoiodo-Tyr in **12** yielded **14,** with decreased affinity for sst$_4$ and some loss of sst$_4$ selectivity. Because of the positive results obtained with the introduction of Aph at position 7 to yield **9** and **10**, we

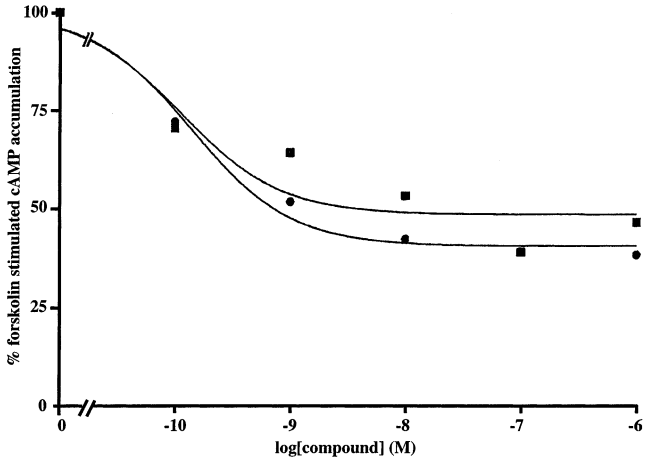


Figure 1. Effect of various concentrations of SRIF-28 and the sst_4-selective agonist **12** on forskolin-stimulated cAMP accumulation in CCL39 cells expressing sst_4. Concentration−response curves were obtained with increasing concentrations of SRIF-28 (●) and **12** (■). Data are expressed as % forskolin-stimulated cAMP accumulation. The plot represents the mean of two independent experiments. Analogue **12** behaves as an agonist.

used this substitution in addition to a DCys^3 and Tyr^2 to yield **15** and **16**. It is clear that this particular combination is not favorable, since both **15** and **16** have lower affinity for all receptors than their parents **9** and **10**.

To determine the importance of having Tyr in position 7 for high affinity and selectivity, we synthesized **17** and **18**, where Phe in position 11 rather than in position 7 was substituted with Tyr. Tyr^{11} substitution was accompanied by some decrease in binding affinities. For example, H-c[DCys-Phe-Phe-L-*threo*-β-Me2Nal-Lys-Thr-Tyr-Lys]-OH (**18**) showed a 4-fold decrease in IC_{50} for sst_4 ($IC_{50} = 11.8 \pm 3$ nM) relative to the most potent and sst_4-selective **12** ($IC_{50} = 3.5 \pm 0.5$ nM) and a slight decrease in sst_4 selectivity. Nonradioactive monoiodination at Tyr^{11} of **18** (obtained by substituting Tyr by monoiodo-Tyr in a de novo synthesis), to yield **20**, tripled its IC_{50} to 30 ± 5.1 nM (**20**) and caused the corresponding loss of selectivity for sst_4. On the other hand, similar monoiodination of **17** yielded **19**, with high affinity ($IC_{50} = 26$, 9.7, 1.8, and 23 nM for sst_2−sst_5, respectively) for all receptors but sst_1. We have no explanation for the deleterious effect of monoiodination in **20** as compared to a favorable effect in **19**, where the only difference is a single inversion of chirality at position 8. Since we had shown that carbamoylation of the N-terminus of sst_3-selective analogues resulted in some significant improvement in affinity, this modification was also introduced at the N-terminus of **17**−**20** with no significant improvement (data not shown).

To conclude, we designed and characterized SRIF analogues such as **6** and **12** that are highly potent, sst_4-selective peptide ligands. Analogues **6** and **12** have >50- and 100-fold selectivity for sst_4 over the other four sst's, respectively, while retaining binding affinity comparable to that of SRIF-28 for sst_4. A tyrosine residue at position 7 or 11 may theoretically be radioiodinated (**14**, **20**); however, sst_4 binding affinity and selectivity are affected to the extent that the compound is unlikely to fulfill the criteria of an optimal sst_4 radioligand. Clearly, the threo configuration is favored over the erythro, and the L-configuration of the α-carbon of *threo*-β-Me2Nal is favored over the D-configuration for optimization of sst_4 selectivity. This series of novel analogues having a *threo*-L-β-Me2Nal at position 8, combined with a Tyr or Aph substitution at position 7 in the ODN-8 octapeptide scaffold, demonstrates that sst_3 receptor selectivity can be completely biased toward sst_4 selectivity. Therefore, the necessity or the advantage of having a D-amino acid at position 8 of SRIF analogues should be re-evaluated. These findings offer promising new leads for the design of other novel, potent and sst_4-selective SRIF analogues (see the following paper).[69] High-affinity sst_4-selective ligands will be of significant help in broadening our knowledge of sst_4 tissue distribution and biological role in the endocrine, exocrine, and nervous systems. Additionally, we show that such constrained analogues are of interest for the determination of a consensus bioactive conformation of sst_4-selective ligands using NMR spectroscopy (see the following paper).[70]

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: AA, amino acid; Aph, 4-aminophenylalanine; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; Z(2Br), 2-bromobenzyloxycarbonyl; Z(2Cl), 2-chlorobenzyloxycarbonyl; Cbm, carbamoyl (NH_2−CO−); CZE, capillary zone electrophoresis; DCHA, dicyclohexylamine; DIC, *N*,*N*′-diisopropylcarbodiimide; DIPEA, diisopropylethylamine; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; (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; 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 of Horiki et al.[71] All Boc-N$^\alpha$-protected amino acids with side-chain protection {Cys(Mob), Lys[Z(2Cl)], 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)[72] and Boc-β-Me2Nal, which were synthesized in our laboratory. Diethyl acetamidomalonate, HBr gas, quinine, sodium, and 2-vinylnaphthalene were purchased from Aldrich Chemical Co. (Milwaukee, WI). Di-*tert*-butyl dicarbonate was purchased from Novabiochem (San Diego, CA). Trypsin, carboxypeptidase B, and carboxypeptidase A were the products of Roche Diagnostics Corp. (Indianapolis, IN). All reagents and solvents were ACS grade and were used without further purification.

Synthesis of β-Methyl-3-(2-naphthyl)alanine (β-Me2Nal) and Its Boc-N$^\alpha$-Protected Derivatives. (a) Synthesis of 2-(1-Bromoethyl)naphthalene. Bromine gas (39.4 g, 486 mmol) was bubbled into a solution of 2-vinylnaphthalene (75 g, 486 mmol) in benzene (100 mL) over a period of 7−8 h at 55−60 °C. The solution was cooled to room temperature, and the benzene with the small excess of HBr was removed under vacuum, yielding the crude of 2-(1-bromoethyl)naphthalene (105 g, 91%) that was used without further purification. $C_{12}H_{11}Br$: MS 235.12

(b) Synthesis of 2-Acetylamino-2-(1-naphthalen-2-yl-ethyl)malonic Acid Diethyl Ester. 2-(1-Bromoethyl)naphthalene (105 g, 446 mmol) in anhydrous ethanol (150 mL) was added to a stirred solution of diethyl acetamidomalonate (97.6 g, 450 mmol) previously reacted with sodium ethylate (sodium metal, 10.3 g, 448 mmol) in anhydrous ethanol (400 mL). The reaction mixture was stirred at 40 °C overnight. After removal of two-thirds of the solvent in vacuo and refrigeration over-

night, the precipitated product and NaBr crystals were collected by filtration. The solid was washed with cold ethanol and distilled water (to eliminate NaBr), yielding 2-acetylamino-2-(1-naphthalen-2-ylethyl)malonic acid diethyl ester (112 g, 67%) as white crystals: mp 148−150 °C (melting points were measured in open capillaries on a Thomas-Hoover Unimelt melting point apparatus and are uncorrected). $C_{21}H_{25}NO_5$: MS 371.44.

(c) Synthesis of the Mixture of *erythro*-(2*S*,3*S* and 2*R*,3*R*)-*β*-Me2Nal·HCl and *threo*-(2*S*,3*R* and 2*R*,3*S*)-*β*-Me2Nal·HCl. A mixture of 6 N HCl/acetic acid (1:1, 1000 mL) and 2-acetylamino-2-(1-naphthalen-2-ylethyl)malonic acid diethyl ester (230 g, 619 mmol) was refluxed for 8 h and then evaporated to one-third of its volume. The precipitated crystals were collected by filtration and recrystallized from 200 mL of ethanol/water (1:1) to yield the mixture of *erythro*-(2*S*,3*S* and 2*R*,3*R*)-*β*-Me2Nal·HCl and *threo*-(2*S*,3*R* and 2*R*,3*S*)-*β*-Me2Nal·HCl (150 g, 91%): mp 192−202 °C (dec).

(d) Separation of the Mixture of *erythro*-(2*S*,3*S* and 2*R*,3*R*)-*β*-Me2Nal·HCl and *threo*-(2*S*,3*R* and 2*R*,3*S*)-*β*-Me2Nal·HCl by Fractional Crystallization. The mixture of the isomers (100 g, 377 mmol) was dissolved in a boiling water (100 mL) and ethanol (200 mL) mixture, and the solution was allowed to crystallize overnight. Crystals were removed by filtration, resulting in a product containing about 70% of erythro enantiomers. Several recrystallizations of the erythro enantiomers from a 1:1 mixture of water and ethanol afforded 27 g (54%) of this racemate with a purity >95%, determined by CZE: mp 205−213 °C (dec). $C_{14}H_{15}NO_2$·HCl: MS FAB *m*/*e* obsd, 230.17 (M + H); calcd, 230.12 (M + H) + 36.45. ^{1}H NMR: $J_{H_\alpha-H_\beta}$ = 7.75 Hz. The mother liquors of the erythro enantiomers were concentrated to one-third of their volume, and ethanol (20 mL) was added before the pH was adjusted to 6.5 with ammonium hydroxide. The threo racemate precipitated, but this product contained about 20% of the erythro racemate. Several recrystallizations from a 1:1 mixture of water and ethanol afforded 6.5 g (16%) of the threo racemate with a purity of >95%, determined by CZE: mp 225−228 °C (dec). $C_{14}H_{15}NO_2$·HCl: MS FAB *m*/*e* obsd, 230.15 (M + H); calcd, 230.12 (M + H) + 36.45. ^{1}H NMR: $J_{H_\alpha-H_\beta}$ = 5.36 Hz. The configuration of the diastereomers was determined by reference to the relationships between the configurations and the coupling constants between C_α−H and C_β−H, determined by ^{1}H NMR spectra. The samples of the isomers were prepared in D_2O + 5% NaOD, and ^{1}H NMR spectra were recorded on a Bruker NMR spectrometer, operating at 500 MHz. Diastereomers with the larger coupling constant are the *erythro*-(2*S*,3*S* and 2*R*,3*R*)-*β*-Me2Nal, and diastereomers with the smaller coupling constant are the *threo*-(2*S*,3*R* and 2*R*,3*S*)-*β*-Me2Nal.[33,61] The purity of the diastereomers was determined by CZE performed on a Beckman P/ACE System 2050, controlled by an IBM Personal System/2 model 50Z, connected to a ChromJet integrator. CZE analysis employed a field strength of 20 kV at 30 °C, with a buffer of 100 mM sodium phosphate, pH 2.5, on a Beckman eCAP fused silica capillary (75 *μ*m i.d. × 50 cm length). Amino acids were dissolved in 0.1% TFA at a concentration of 100 *μ*g/mL and applied by pressure injection. A baseline separation (difference in migration time of 1 min) of the threo and erythro racemates was accomplished in less than 25 min. The threo racemate migrated faster than the erythro one. Detection of >0.5% of either the erythro or the threo racemate was readily achieved under these conditions.

(e) Separation of *erythro*-(2*R*,3*R* and 2*S*,3*S*)-*β*-Me2Nal Enantiomers as Their N$^\alpha$-*tert*-Butoxycarbonyl Derivatives with Quinine Resolving Agent. The mixture of the erythro enantiomers (33.7 g, 127 mmol) was converted to Boc-N$^\alpha$-*erythro*-(2*R*,3*R* and 2*S*,3*S*)-*β*-Me2Nal using Boc-anhydride under basic conditions. To a solution of the isolated oil (41 g, 125 mmol) in anhydrous ethanol (70 mL) was added a hot solution of quinine (20.4 g, 63 mmol) in anhydrous ethanol (80 mL). After 12 h at room temperature, Boc-N$^\alpha$-*erythro*-2*R*,3*R*-*β*-Me2Nal quinine salt crystallized (35 g, 84%). Recrystallization from ethyl acetate afforded the desired product (22.1 g, 53%). The Boc protecting group was removed with TFA prior to TLC on Chiralplate reversed-phase silica gel plates, impregnated with a chiral selector (a proline derivative) and copper(II) ions (Macherey-Nagel Co., FRG) using an acetonitrile/methanol/water (4:1:1) solvent system to show an optical purity >95%: R_f = 0.4; mp 142−145 °C. For quinine salt, $C_{19}H_{23}NO_4$ + $C_{20}H_{24}N_2O_2$: MS FAB *m*/*e* obsd, 329.90, 324,88, 654.30 (M + H); calcd, 330.17 (M + H) + 324.18 (654.36). After conversion of the quinine salt to free acid, mp 116−118 °C. $[\alpha]^{25}_D$ = −6.5° (*c* = 1; AcOH) and $[\alpha]^{25}_D$ = +6° (*c* = 2; DMF) were measured on a Perkin-Elmer 241 polarimeter at 589 nm; the Clough−Lutz−Jirgensen empirical rule was used to determine the absolute configuration. The molecular rotation is more negative in acetic acid than in DMF, indicating that this is the D-amino acid.[73]

To the mother liquor of Boc-*erythro*-D-*β*-Me2Nal quinine salt was added another 0.5 equiv of quinine (20.4 g, 63 mmol) in anhydrous ethanol (70 mL). Addition of diethyl ether to this solution facilitated crystallization. The product was recrystallized from ethyl acetate, yielding Boc-*erythro*-L-*β*-Me2Nal quinine salt (26 g, 56.4%). Optical purity, estimated with TLC, was >80%; R_f = 0.6 (prior to TLC, the Boc group was removed with TFA); mp 180−184 °C. For quinine salt, $C_{19}H_{23}NO_4$ + $C_{20}H_{24}N_2O_2$, MS FAB *m*/*e* obsd, 329.99, 324.90, 654.3 (M + H); calcd, 330.17 (M + H) + 324.18 (654.36). Conversion of the quinine salt to free acid yielded a thick oil, for which no melting point was measured. $[\alpha]^{25}_D$ = +5.8° (*c* = 1; AcOH) and $[\alpha]^{25}_D$ = −7.5° (*c* = 2; DMF). The molecular rotation is more positive in acetic acid than in DMF, indicating that this is the L-amino acid.[73]

(f) Separation of *threo*-(2*R*,3*S* and 2*S*,3*R*)-*β*-Me2Nal Enantiomers as Their *tert*-Butoxycarbonyl Derivatives. The mixture of the pure threo enantiomers (5 g, 21.8 mmol) was converted to Boc-*threo*-(2*R*,3*S* and 2*S*,3*R*)-*β*-Me2Nal (6.6 g, 92%). Separation of the two Boc-protected isomers following the same protocol that was used for the separation of the erythro isomers, fractional crystallization of the diastereomer salts with quinine, failed. The racemate mixture was converted to DCHA salt form to facilitate crystallization, yielding 9.2 g (82.6%) of the mixture of the two enantiomers as the DCHA salts: mp 147−148 °C. $C_{19}H_{23}NO_4$ + $C_{12}H_{23}N_1$: MS FAB *m*/*e* obsd 181.95, 330.10, 511.10 (M + H); calcd 330.17 (M + H) + 181.18 (511.35)

Boc-N$^\alpha$-*threo*-(2*R*,3*S*) and Boc-N$^\alpha$-*threo*-(2*S*,3*R*) enantiomers were used in their racemate form in the peptide synthesis, and the *threo*-(2*R*,3*S*)- and the *threo*-(2*S*,3*R*)-*β*-Me2Nal-containing peptide mixtures were separated by RP-HPLC. The configurations of the *β*-methyl amino acids in the peptides were determined by enzymatic hydrolysis. A similar approach with the erythro isomer-containing analogues gave further evidence to our assumption based on the Clough−Lutz−Jirgensen empirical rule.[73]

Peptide Synthesis. Peptides were synthesized by the solid-phase approach either manually or on a CS-Bio peptide synthesizer, model CS536.[74] 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 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[62] on a 5-cm × 30-cm cartridge, packed in the laboratory with reversed-phase 300 Å Vydac C_{18} silica (15−20 *μ*m 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 above. Each peptide is >90% pure 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% TFA and 50% acetonitrile. The observed monoisotopic $(M + H)^+$ values of each peptide corresponded with the calculated $(M + H)^+$ values.

Determination of the Stereochemistry of *threo*-β-Me2Nal in the Peptides. A very good separation of the *threo*-D- from the *threo*-L-β-Me2Nal-containing peptides was obtained by RP-HPLC. The absolute configuration at the α-carbon of the *threo*-β-Me2Nal in **11** (eluting faster from the RP-HPLC C_{18} column) and **12** (Table 1) was determined by enzymatic hydrolysis with trypsin, carboxypeptidase B, and carboxypeptidase A in three consecutive steps. Trypsin was able to open the ring structure of compound **11** (partially in >4 days) and compound **12** (within 2 h) after the lysine residue, to yield the linear free acids, which eluted earlier on the RP-HPLC and showed an 18-mass-unit increase. We further hydrolyzed the linear derivative of **12** with carboxypeptidase B, which quantitatively cleaved the C-terminus lysine within an hour, as shown by MS and a shift in RP-HPLC retention time toward the more hydrophobic region. Finally, this product was completely digested with carboxypeptidase A, suggesting that all amino acids in the remaining sequence were of the L-configuration. One of the free amino acids (β-Me2Nal) could be identified as one of the hydrolysis products by RP-HPLC. Using identical RP-HPLC conditions, the configuration of *threo*-β-Me2Nal in the rest of the analogues was deduced from the above observation; that is, the faster-eluting analogue on RP-HPLC contained the *threo*-D-β-Me2Nal enantiomer and the slower-eluting, more hydrophobic peptide contained the *threo*-L-β-Me2Nal enantiomer in the sequence. This assumption was confirmed in all cases by the different rates of hydrolysis of each pair of diastereomers using trypsin.

Receptor Autoradiography. Cells stably expressing the cloned five human sst's were grown as described previously.[64] All cell culture reagents were supplied by Gibco BRL, Life Technologies (Grand Island, NY). The receptor autoradiographical experiments were performed as reported previously,[64] using ^{125}I-[Leu^8,DTrp^{22},Tyr^{25}]SRIF-28 as tracer.

Adenylate Cyclase Activity. Modulation of forskolin-stimulated adenylate cyclase activity was determined using a radioimmunoassay measuring intracellular cAMP levels by competition binding.[75] sst_4-expressing cells were subcultured in 96-well culture plates at 2 × 10^4 cells/well and grown for 24 h. Culture medium was removed from the wells, and 100 mL 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 3 mM 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 (RPA 538; Amersham Biosciences, Little Chalfont, UK), according to the instructions from the manufacturer (Amersham, Aylesbury, 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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