Highly Potent and Subtype Selective Ligands Derived by *N*-Methyl Scan of a Somatostatin Antagonist

W. G. Rajeswaran,[†] Simon J. Hocart,[†] William A. Murphy,[†] John E. Taylor,[‡] and David H. Coy*[,†]

Peptide Research Labs, SL 12, Department of Medicine, Tulane University Health Sciences Center, 1430 Tulane Avenue, New Orleans, Louisiana 70112, and Biomeasure Inc., Milford, Massachusetts 01757

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The search for synthetic peptide analogues of somatostatin (SRIF) which exhibit selective affinities for the five known receptor subtypes (sst_{1-5}) has generated a large number of potent agonists. Some of these agonists display good subtype selectivities and affinities for the subtypes 1, 2, 3, and 5, including analogues created by *N*-methyl amino acid substitutions in a standard octapeptide analogue format. We have now extended this peptide backbone N-methylation approach to a potent somatostatin receptor antagonist series using the antagonist Cpa-cyclo-(DCys-Pal-DTrp-Lys-Thr-Cys)-Nal-NH_2 **9** reported[21] from this laboratory as the lead structure. Synthetic analogues were tested for their ability to inhibit somatostatin-stimulated GH release from rat pituitary cells in culture and to displace ^{125}I-labeled somatostatin from CHO cells transfected with the five known human somatostatin receptors. Several interesting observations resulted from the study. N-Methylation at the Lys[9] residue (**5**) increased the rat GH release inhibitory potency nearly 4-fold to 0.73 nM but resulted in little change in the binding affinity for human type 2 receptor. This analogue also had a high affinity of 5.98 nM for sst_5 receptor (compared to 1.4 nM for somatostatin itself) and is the first antagonist analogue to be reported with high affinity for sst_5. It also had high potency on in vitro inhibition of sst_5 mediated intracellular calcium mobilization. These results were considered surprising, since the Lys[9] residue has long been considered to constitute the active center of somatostatin, important both for receptor binding and activation, and suggests important conformational differences between D-Cys[9] somatostatin antagonists and normal agonist structures. More modifications were carried out on this analogue with the aim of improving antagonist potency and/or specificity. Tyr[7] substitution of **5** resulted in an analogue, which had the highest affinity in the series for $hsst_2$ (K_I 5.51 nM) and an extraordinarily low IC_{50} of 0.53 nM in the rat pituitary cell assay. However, this analogue lost considerable affinity for sst_5 relative to analogue **5**. Analogue **16** with DTrp[12] at C-terminus had the highest affinity for $hsst_2$, however, the IC_{50} in the rat GH release assay was only 11.6 nM. Replacement of Lys[9] in **9** with Dab[9] gave **11** which displayed high binding affinity for sst_3, and it was also quite selective for that receptor. Both the sst_3 and sst_5 antagonists should be of value in assigning the physiological roles to type 3 and 5 receptor, respectively.

Introduction

Somatostatin (SRIF), a tetradecapeptide discovered by Brazeau et al.,[1] has been shown to have potent inhibitory effects on various secretory processes in tissues such as pituitary, pancreas, or gastrointestinal tract. SRIF also acts as a neuromodulator in the central nervous system.[2] These biological effects of SRIF, all inhibitory in nature, are elicited through a series of G protein coupled receptors, of which five different subtypes have been characterized (sst_1−sst_5).[3] These five subtypes have similar affinities for the endogenous SRIF ligands but have differing distribution in various tissues.[4] The development of potent, smaller SRIF agonists led to the discovery of differing affinities of the various truncated ligands for the different subtypes.[5,6] It has been demonstrated that only the Trp[8]-Lys[9] residue pair is required for receptor recognition and bioactivity.[7−9] This crucial residue forms part of a β-bend which is usually stabilized via substitution of D- for L-Trp, cyclization of the backbone, a disulfide bridge, or all constraints.[10] One unintended consequence of such structural simplification, carried out before the discovery of multiple receptor subtypes, was the loss of broad spectrum binding affinity. This is typified by the high type 2 but low type 1, 3, 4, and 5 affinities of peptides in the octreotide (sandostatin) series.[11] Thus, the many basic biological studies with this type of analogue failed to detect effects mediated by all but one of the somatostatin receptor types. Since then, much work in our laboratory has gone into the reintroduction of broader spectrum binding[12] into small, biologically stable peptides on one hand and the development of peptides and peptidomimetics[13] with discrete specificity for a particular receptor, on the other.

Thus far, of the five receptor subtypes for somatostatin, only sst_2 and sst_5 have been associated with specific physiological functions. Type 2 has a predominant role in mediating the release of GH as evidenced by 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.[6] Inhibition of insulin secretion is thought to be

* To whom correspondence should be addressed. Phone: (504) 584-3584. Fax: (504) 584-3586. E-mail: dcoy@tulane.edu.
[†] Tulane University Health Sciences Center.
[‡] Biomeasure Inc.

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Table 1. *N*-Methyl Analogues and Analytical Data

		mass spectrum ($M - H^+$)		HPLC[c]	
no.	N-Me sequence	calcd[a]	obsd[b]	(t_{R-1})[d]	(t_{R-2})[e]
1	NMeCpa-cyclo(DCys-Pal-DTrp-Lys-Thr-Cys)-Nal-NH_2	1178.7	1179.2	18.3	19.3
2	Cpa-cyclo(NMeDCys-Pal-DTrp-Lys-Thr-Cys)-Nal-NHMe	1192.7	1193.4	19.6	19.3
3	Cpa-cyclo(DCys-NMePal-DTrp-Lys-Thr-Cys)-Nal-NH_2	1178.7	1178.9	20.3	22.5
4	Cpa-cyclo(DCys-Pal-NMeDTrp-Lys-Thr-Cys)-Nal-NH_2	1178.7	1179.2	17.9	17.2
5	Cpa-cyclo(DCys-Pal-DTrp-NMeLys-Thr-Cys)-Nal-NH_2	1178.7	1178.9	19.2	18.7
6	Cpa-cyclo(DCys-Pal-DTrp-Lys-NMeThr-Cys)-Nal-NH_2	1178.7	1179.3	17.4	15.1
7	Cpa-cyclo(DCys-Pal-DTrp-Lys-Thr-NMeCys)-Nal-NH_2	1178.7	1179.0	18.5	16.7
8	Cpa-cyclo(DCys-Pal-DTrp-Lys-Thr-Cys)-NMeNal-NH_2	1178.7	1179.0	20.3	19.0
9	Cpa-cyclo(DCys-Pal-DTrp-Lys-Thr-Cys)-Nal-NH_2	1164.8	1164.7	17.2	17.2
10	Cpa-cyclo(NMeDCys-Pal-DTrp-Lys-Thr-Cys)-Dip-NHMe	1218.9	1218.9	21.9	20.8
11	Cpa-cyclo(DCys-Pal-DTrp-Dab-Thr-Cys)-Nal-NH_2	1136.6	1137.2	18.0	16.9
12	Cpa-cyclo(DCys-Pal-DTrp-DDap-Thr-Cys)-Nal-NH_2	1122.5	1123.4	18.8	19.8
13	Cpa-cyclo(DCys-Pal-NMeDTrp-NMeLys-Thr-Cys)-Nal-NH_2	1192.7	1192.3	19.9	19.7
14	Cpa-cyclo(DCys-Tyr-DTrp-NMeLys-Thr-Cys)-Nal-NH_2	1193.8	1193.6	24.9	23.1
15	Tfm-cyclo(DCys-Pal-DTrp-NMeLys-Thr-Cys)-Nal-NH_2	1212.2	1212.2	21.4	20.7
16	Cpa-cyclo(DCys-Pal-DTrp-NMeLys-Thr-Cys)-DTrp-NH_2	1167.8	1168.0	16.6	14.9
17	Nal-cyclo(DCys-Pal-DTrp-NMeLys-Thr-Cys)-DTrp-NH_2	1183.2	1183.5	18.0	16.4
18	Pal-cyclo(DCys-Pal-DTrp-NMeLys-Thr-Cys)-DTrp-NH_2	1135.0	1134.8	11.5	9.8

[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, $\lambda = 215$ nm) retention times (min). Each compound was found to have a purity of >98% by HPLC. [d] HPLC elution system: A, 0.1% TFA; B, 0.1% TFA in 80% MeCN; 20% B to 50% 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; 10% D to 50% D at 1% min^{-1} and 1.5 mL min^{-1}.

mediated through sst_5.[14] However, elucidating the physiological role of each of the receptor subtypes would be greatly enhanced by the development of receptor subtype-specific antagonists.[4] Also SRIF antagonists might provide a novel route to increasing endogenous levels of some hormones, notably growth hormone and insulin.

One interesting approach, just made easier with new solid-phase chemistry,[15] was the introduction of peptide backbone constraint imposed by N-alkylation. This could yield useful information about bioactive conformation since *N*-methyl amino acid substitutions have often been used to increase the potency or selectivity of a peptide ligand.[16,17] This modification largely restricts the affected residue and the amino acid preceding it to an extended conformation,[18] and it additionally blocks potential intramolecular hydrogen bonding sites and also proteolytic enzyme cleavage sites, thus potentially enhancing pharmacokinetic properties of a peptide. Not all *N*-methyl amino acids are commercially available, and their synthesis is time-consuming.[13] In this paper, we have described the binding affinity, for the five cloned human somatostatin receptors, of N-methylated somatostatin antagonists and also their activity on GH release from rat pituitary cells, which is a subtype 2 receptor mediated event.[6]

Results and Discussion

As an extension to the *N*-methyl scanning strategy applied to shortened somatostatin agonist analogues,[19] the technique has now been examined for development of subtype specific somatostatin antagonists,[20,21] using the potent somatostatin antagonist Cpa-cyclo(DCys-Pal-DTrp-Lys-Thr-Cys)-Nal-NH_2[20,21] (**9**) as the base structure. The binding affinities (K_d, nM) of all somatostatin analogues were determined using their concentration-dependent displacement of ^{125}I-radiolabeled Tyr^{11}-somatostatin from membranes isolated from CHO cells transfected with the corresponding human somatostatin receptors and are shown in Table 2. For reference, the binding affinities of SRIF-14 and SRIF-28 in the same system are included. SRIF-28 displays particularly high affinity for type 5 receptors compared to somatostatin-14.[14]

SRIF analogue **9** was alkylated at every residue by a solid-phase procedure[15] while being assembled on methylbenzhydrylamine resin. After the *tert*-butoxycarbonyl (Boc) group was removed at the desired N-methylation site, the free amine of the resin bound peptide was protected using *o*-nitrobenzenesulfonyl chloride and collidine in dichloromethane. Then the amide N−H of *o*-nitrobenzenesulfonamide (*o*-NBS) was selectively deprotonated by the strong, hindered, nonionic base MTBD and methylated using methyl 4-nitrobenzenesulfonate in DMF. The methylated sulfonamide was deprotected by β-mercaptoethanol and DBU in DMF. The subsequent amino acid was coupled two times using TBTU/DIPEA instead of DIC (Scheme 1). The structures and physiochemical characteristics are given in Table 1.

The above methylation procedure worked well for all of the residues, except for D-Cys^6. Methylation at this site only yielded dimethylated derivative **2**. This could be attributed to the spatial orientation of the *o*-NBS group of the protected D-Cys^6. The nitro group in this peptide is oriented in such a way that it H-bonds with the amide NH of the resin bound Nal^{12}. This increases the acidity of the amide NH of (Nal^{12}) and facilitates deprotonation by the added base, MTBD. It was conjectured that a bulky α-substituent at the C-terminus might hinder the methylation at the C-terminus amide. Accordingly, Nal^{12} was replaced by Dip^{12}. Unfortunately, N-methylation of D-Cys^6 residue of this peptide only yielded the dimethylated compound **10**. However, methylation after replacing the D-Cys^6 with Cys^6 gave the expected monomethylated (N-$MeCys^6$) peptide.

N-Methylation of **9** at Cpa^5 decreased the affinity for type 2 and 3 while increasing the affinity for type 5 receptor. As a simple measure of the conformational changes, which occur with methylation, the molecular-mechanical energy of each analogue was compared with the energy of the parent compound. These energy changes are driven by the rearrangements required to

Table 2. Binding Affinities (K_I) for the Cloned Human sst_{1-5} Receptors and Antagonist Data

peptide no.	N$^{\alpha}$-methylation site	K_I[a] ± SEM (nM)					antagonist IC_{50} ± SEM (n)[b](nM)
		$hsst_1$	$hsst_2$	$hsst_3$	$hsst_4$	$hsst_5$	
SRIF-14	N/A[c]	2.0 ± 0.35	0.25 ± 0.03	1.2 ± 0.2	2.0 ± 0.3	1.4 ± 0.3	N/A[c]
SRIF-28	N/A[c]	1.9 ± 0.4	0.31 ± 0.06	1.3 ± 0.3	5.4 ± 2.5	0.4 ± 0.1	N/A[c]
1	Cpa^5	1000	36 ± 7.6	330 ± 126	1000	40.1 ± 18.8	7.8 ± 2.7
2	D-Cys^{6d}	1000	89.0 ± 8.0	576 ± 47	1000	106 ± 36	nd[e]
3	Pal^7	1000	189.0 ± 35	450 ± 132	1000	1000	nd[e]
4	D-Trp^8	1000	51.8 ± 2.6	390 ± 114	1000	93 ± 17.7	nd[e]
5	Lys^9	1000	17.1 ± 5.5	66.0 ± 5.8	1000	5.98 ± 0.91	0.73 ± 0.33
6	Thr^{10}	395 ± 202	1000	315 ± 12.5	1000	88.5 ± 45.7	nd[e]
7	Cys^{11}	1000	810	68.7 ± 4.7	575	161 ± 52	nd[e]
8	Nal^{12}	1000	197 ± 55	1000	1000	1000	nd[e]
9	N/A[c]	1395	12.1 ± 1.9	38.2 ± 2.4	1000	140 ± 4.6	2.6 ± 0.7
10	D-Cys^{6d}	1000	117 ± 24.6	584 ± 305	1000	766 ± 110	nd[e]
11	N/A[c]	1000	317 ± 28	23.6 ± 2.02	1000	1000	102 ± 80
12	N/A[c]	1000	1000	1000	1000	1000	nd[e]
13	DTrp^8 and Lys^9	1000	9.33 ± 0.62	140 ± 10.4	1000	112 ± 19	2.5 ± 0.20
14	Lys^9	1000	5.51 ± 1.85	115.1 ± 16.9	1000	70.7 ± 25.8	0.53 ± 0.17
15	Lys^9	1000	11.3	40.2	1246	45.5	nd[e]
16	Lys^9	1000	5.45 ± 0.3	91.4 ± 11.9	1000	101 ± 14.1	11.6 ± 4.2
17	Lys^9	1000	27.3 ± 1.45	148 ± 13.2	1000	176 ± 65.1	96 ± 13.8
18	Lys^9	1000	24.7 ± 1.61	537 ± 44.8	1000	313 ± 4.1	287 ± 138

[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] Not applicable. [d] Compounds **2** and **10** are different peptides. [e] Not determined.

Scheme 1

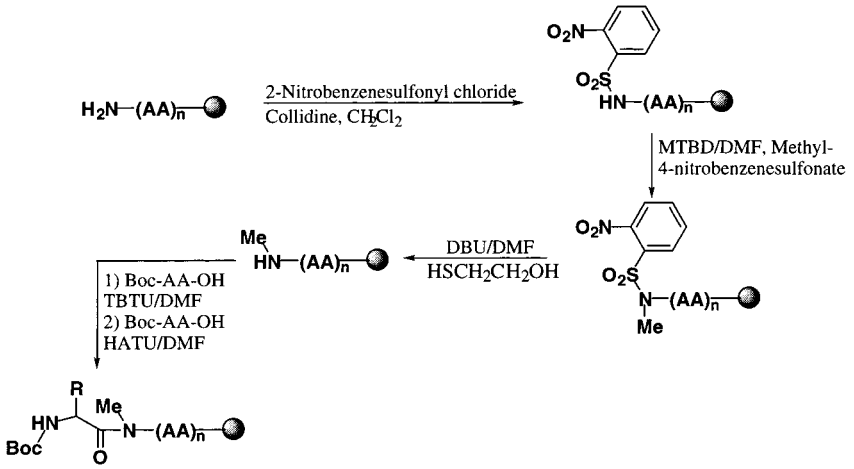

Table 3. Kollman All Atom Energy Change on Sequential Methylation of Each Residue of Cpa-cyclo(DCys-Pal-DTrp-Lys-Thr-Cys)-Nal-NH_2 (Analogue **9**) in Each of the Three Solution Conformations Expressed as kcal mol^{-1}

analogue no.	methylation site	I	II	III
1	NMeCpa^5	−2	0.4	−1
2	NMedCys^6, NHMe	17	12	31
3	NMePal^7	7	17	6
4	NMedTrp^8	5	6	5
5	NMeLys^9	6	6	5
6	NMeThr^{10}	16	10	12
7	NMeCys^{11}	12	14	23
8	NMeNal^{12}	4	19	7

accommodate the steric bulk of the methyl group and are given in Table 3. The terminal amino group is relatively accessible, and the addition of steric bulk by methylation was accompanied by no significant change in the energy of the molecular models. N-Methylation at D-Cys^6, Pal^7, or D-Trp^8 had decreased affinity for type 2 and 3 receptors. In the case of D-Cys^6 and Pal,^{7} methylation was accompanied by major perturbations to the conformation of the backbone, with the elimination of some transannular hydrogen bonding sites. However, N-methylation at Lys^9 had a remarkable effect on both binding and antagonist activity. The methylation sites in D-Trp^8 and Lys^9 are outside the backbone ring, are accessible, and tolerate the steric bulk of methylation with little change in the overall conformation as shown by the small change in energy on methylation at these sites (see Table 3). However, the addition of methyl groups to either position adds a large steric component in the critical β-bend part of the molecule responsible for receptor recognition. Although N-Me-Lys^9 **5** had slightly less affinity, compared to the parent peptide **9** for sst_2, it is almost 4 times more potent than the parent peptide in the GH release assay and also had high binding affinity for the type 5 receptor. It had an IC_{50} of 22.6 ± 7.6 nM in in vitro inhibition of type 5 mediated intracellular calcium mobilization (Figure 1). Since both the type 2 and type 5 receptors are involved in inhibitory control of GH release[22] in human pituitary cells, it is possible that this type of dual receptor antagonist might offer a better means of increasing tonic GH levels via blockade of both 2 and 5 somatostatin receptors. Also, type 5 receptors

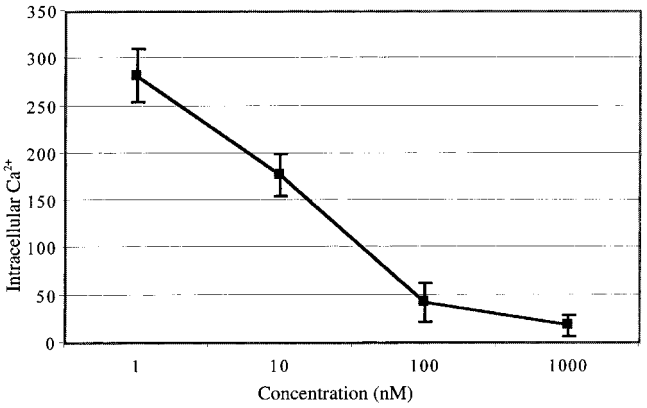

Figure 1. In vitro inhibition of $hsst_5$ mediated intracellular Ca^{2+} mobilization.

appear to mediate the inhibitory effects of endogenous somatostatin on pancreatic insulin release,[23] suggesting that a type 5 antagonist could provide a means of increasing insulin secretory responses via blockade of endogenous somatostatin binding which is perhaps of therapeutic interest in early stage type 2 diabetes.

Since methylation at the putative active motif (D-Trp^8-Lys^9) resulted in a biologically more active compound, it was decided to explore the effects of the N-Me group at this site with a variety of variations on either side of the active center. N-Methylation at both D-Trp^8 and Lys^9 gave the peptide **13**, which had slightly better affinity for type 2 receptor and potency in the GH release assay. Replacement of Pal^7 by Tyr^7 in **5** gave the peptide **14** with 3 times increased affinity for the sst_2 receptor and also with more specificity. The IC_{50} of this compound for inhibition of growth hormone release was 0.53 nM. To the best of our knowledge, this is the most potent antagonist for the sst_2 receptor so far reported since it is 2 times more potent than its parent compound Cpa-cyclo[DCys-Tyr-DTrp-Lys-Thr-Cys]-Nal-NH_2 previously reported from this laboratory.[21] Substitution of Tfm,[5] at the N-terminus, for Cpa^5 resulted in improved affinity for type 2 and 3 receptors but considerable loss of affinity for type 5 receptors. D-Trp^{12} at the C-terminus of **5** showed increased affinity for type 2 but decreased potency for rat GH release activity. N-Terminus replacement of **16** either with Nal^5 or Pal^5 resulted in appreciable loss of GH release potency.

Analogue **5** showed that the amide N−H of the active center could be modified to yield a compound with high potency. Keeping this in mind, it was decided to reexamine the structure−activity relationships of the Lys side chain. Replacement of Lys^9 with shorter side chain Dab^9 yielded analogue **11** with high type 3 selectivity but reduced affinity for type 2 and type 5 receptors. The physiological roles of type 3 somatostatin receptors have not been well characterized although they are particularly heavily distributed throughout the gastrointestinal tract, including gastric smooth muscle cells,[24] so that this selective type 3 antagonist could be a useful tool in this respect. Although several D-Cys^6 analogues behave as an antagonist for sst_2 and sst_5 receptors, at present there is no proof that this motif applied in analogue **11**, which also binds to sst_3, produces an sst_3 antagonist. Further work is in progress to elucidate this point.

These results confirm once more that N^{α}-alkylation is a powerful approach to improving the specificity and potency of peptide−receptor interactions and should be considered equally with the more widely used strategies of D-amino acid, reduced peptide bond, and constrained amino acid substitution. This is now even more readily accomplished synthetically using the new solid-phase alkylation procedure. Further work on peptides, conformationally restricted by N^{α}-alkylation, N^{α}-allylation, and N^{α}-arylation, is in progress in this lab. Attempts will also be made to impose conformational restriction by cyclization using ring closing metathesis techniques on solid phases.

Experimental Section

Abbreviations. The nomenclature for the somatostatin receptor subtypes is in accordance with the recommendations of IUPHAR,[25] in which sst_4 refers to the receptor originally cloned by Bruno et al.,[26] and sst_5 refers to the receptor cloned by O'Carroll et al.[14,27] Abbreviations of the common amino acids are in accordance with the recommendations of IUPAC−IUB.[28] Additional abbreviations: Dab, 2,4-diaminobutyric acid; Dap, 2,3-diaminopropionic acid; Cpa, 3-(4-chlorophenyl)alanine; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; MTBD, 1,3,4,6,7,8-hexahydro-1-methyl-2*H*-pyrimido[1,2-*a*]pyrimidine; Nal, 3-(2-naphthyl)alanine; *o*-NBS, 2-nitrobenzenesulfonyl; Pal, 3-(3-pyridyl)alanine; TBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; Tfm, 4-trifluoromethylphenylalanine.

Materials. 4-Methylbenzhydrylamine hydrochloride resin (0.25 or 0.5 mequiv g^{-1}) was obtained from Advanced ChemTech Inc., Louisville, KY. N^{α}-*tert*-Butyloxycarbonyl (Boc) 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; Lys, 2-chlorobenzyloxycarbonyl; 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 (0.25 or 0.5 mequiv g^{-1}), in 0.25 mmol scale on an Advanced ChemTech (model 200) synthesizer, using the following protocol: deblocking, 40% TFA (2 min, 20 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-diisopropyl carbodiimide 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.

N^{α}-Protection. After deblocking the amino group at the desired methylation site, the resin was suspended in DCM (20 mL). To this suspension, collidine (3 equiv) and *o*-nitrobenzenesulfonyl chloride (3 equiv) were added, and the mixture was shaken using an Advanced ChemTech (model 200) synthesizer for 2 h. Then the resin was subjected to DCM wash (2 washes) and DMF wash (3 washes). Protection was monitored qualitatively by the ninhydrin test.

N^{α}-Methylation. The *o*-nitrobenzenesulfonamide protected resin was suspended in DMF (20 mL), to which MTBD (3 equiv) and methyl 4-nitrobenzenesulfonate or dimethyl sulfate (for Cys^{11}) were added. The mixture was shaken using an Advanced ChemTech (model 200) synthesizer for 0.5 h, and the resin was subjected to DMF wash (four washes).

Deprotection. Once the desired residue was methylated, the resin was again suspended in DMF (20 mL). DBU (3 equiv) and 2-mercaptoethanol (3 equiv) were added to the suspension, and the mixture was agitated for 0.5 h in an Advanced

ChemTech (model 200) synthesizer. Then the resin was thoroughly washed with DMF (five washes).

Peptide Cleavage. The peptides were cleaved from the resin support with simultaneous side chain deprotection by acidolysis using anhydrous hydrogen fluoride containing the scavenger anisole (~30% v/v) for 45 min at 0 °C. The peptides were cyclized in 90% acetic acid (~600 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. 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. 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 linear gradient system was used at a flow rate of 1.5 mL min^{-1}: HPLC-1, A, 0.1% TFA; B, 0.1% TFA in 80% MeCN; 20% B to 50% B at 1% min^{-1}; HPLC-2, C, 5% MeCN in TEAP (0.1 M, pH 3); D, 20% C in MeCN; 10% D to 50% D at 1% min^{-1}. Column eluent was monitored at 215 nm. The retention time and purity of each peptide was assessed by the Rainin Dynamax HPLC Method Manager. Each peptide was found to have a purity of ≥98%. 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 hydrolyzates 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.[29] 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 (six 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 incubation 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 the 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 ($hsst_{1-5}$),[30−33] 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 a 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-minimum essential medium (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} of 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 receptor 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 was 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.

Type 5 Mediated Intracellular Ca^{2+} Mobilization. CHO-K1 cells, expressing the human sst5 receptor, were harvested by incubating in a 0.3% EDTA/phosphate buffered saline solution (25 ° C) and washed twice by centrifugation. The washed cells were resuspended in Hank's-buffered saline solution (HBSS) for loading of the fluorescent Ca^{2+} indicator Fura-2AM. Cell suspensions of approximately 10^6 cells/mL were incubated with 2 μM Fura-2AM for 30 min of 25 °C. Unloaded Fura-2AM was removed by centrifugation twice in HBBS, and the final suspensions were transferred to a spectrofluorometer (Hitachi F-2000) equipped with a magnetic stirring mechanism and a temperature-regulated cuvette holder. After equilibration to 37 °C, the somatostatin peptides were added for measurement of intracellular Ca^{2+} mobilization. The excitation and emission wavelengths were 340 and 510 nm, respectively.

Molecular Modeling. All molecular modeling was performed on a Silicon Graphics Indigo^2 High Impact 10000 computer, using SYBYL 6.6[34] with the Kollman all atom force field.[35] The PDB files for the three solution NMR structures of the initial compound Sandostatin/Octreotide, dPhe^5-c[Cys^6-Phe^7-DTrp^8-Lys^9-Thr^{10}-Cys^{11}]-Thr^{12}-ol (1SOC and 2SOC), were obtained from the PDB database. These structures were imported into SYBYL6.6 and mutated to form the N-methylated compounds based on analogue **9**. The Kollman partial atomic charges were loaded from the monomer dictionary. The structures were optimized by annealing the mutated residue and then by full-energy minimization using the conjugate gradient algorithm to a final root-mean-square (rms) gradient of ≤0.01 kcal mol Å^{-1}. A distance-dependent dielectric function[36] was employed together with the default settings for all the other minimization options.

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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) Epelbaum, J. Somatostatin in the central nervous system: physiology and pathological modifications. *Prog. Neurobiol.* **1986**, *27*, 63−100.

(3) Reisine, T.; Bell, G. I. Molecular properties of somatostatin receptors. *Neuroscience* **1995**, *67*, 777−790.

(4) Reisine, T.; Bell, G. I. Molecular biology of somatostatin receptors. *Endocr. Rev.* **1995**, *16*, 427−442.

(5) Coy, D. H.; Taylor, J. E. Receptor-specific somatostatin analogues: Correlations with biological activity. *Metabolism* **1996**, *34* (1), 21−23.

(6) 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.

(7) 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.

(8) Nutt, R. F.; Veber, D. F.; Curely, P. E.; Saperstein, R.; Hirschmann, R. F. Somatostatin analogues which define the role of lysine-9 amino group. *Int. J. Pept. Protein Res.* **1983**, *21*, 66−73.

(9) Veber, D. F.; Holly, F. W.; Nutt, R. F.; Bergstrand, S. J.; Brady, S. F.; Hirschmann, R. Highly active cyclic and bicyclic somatostatin analogues of reduced ring size. *Nature* **1979**, *280*, 512−514.

(10) 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.

(11) Bauer, W.; Briner, U.; Doepfner, W.; Haller, R.; Huguenin, R.; Marbach, P.; Petcher, T. J.; Pless, J. SMS 201-995: A very potent and selective octapeptide analogue of somatostatin with prolonged action. *Life Sci.* **1982**, *31*, 1133−1140.

(12) Woltering, E. A.; O'Dorisio, M. S.; Murphy, W. A.; Chen, F.; Drouant, G. J.; Espenan, G. D.; Fisher, D. R.; Sharma, C.; Diaco, D. S.; Maloney, T. M.; Fuselier, J. A.; Nelson, J. A.; O'Dorisio, T. M.; Coy, D. H. Synthesis and characterization of multiple-tyrosinated, multiple-iodinated somatostatin analogues. *J. Pept. Res.* **1999**, *53*, 201−213.

(13) Rohrer, S. P.; Birzin, E. T.; Mosley, R. T.; Berk, S. C.; Hutchins, S. M.; Shen, D. M.; Xiong, Y.; Hayes, E. C.; Parmar, R. M.; Foor, F.; Mitra, S. W.; Degrado, S. J.; Shu, M.; Klopp, J. M.; Cai, S. J.; Blake, A.; Chan, W. W. S.; Pasternak, A.; Yang, L.; Patchett, A. A.; Smith, R. G.; Chapman, K. T.; Schaeffer, J. M. Rapid identification of subtype-selective agonists of the somatostatin receptor through combinatorial chemistry. *Science* **1998**, *282*, 737−740.

(14) O'Carroll, A.-M.; Raynor, K.; Lolait, S. J.; Reisine, T. Characterization of cloned human somatostatin receptor SSTR5. *Mol. Pharmacol.* **1994**, *46*, 291−298.

(15) Miller, S. C.; Scanlan, T. S. Site-selective N-methylation of peptides on solid support. *J. Am. Chem. Soc.* **1997**, *119*, 2301−2302.

(16) Ali, F. E.; Bennett, D. B.; Calvo, R. R.; Elliott, J. D.; Hwang, S.-M.; Ku, T. W.; Lago, M. A.; Nichols, A. J.; Romoff, T. T.; Shah, D. H.; Vasko, J. A.; Wong, A. S.; Yellin, T. O.; Yuan, C.-K.; Samanen, J. M. Conformationally constrained peptides and semipeptides derived from RGD as potent inhibitors of the platelet fibrinogen receptor and platelet aggregation. *J. Med. Chem.* **1994**, *37*, 769−780.

(17) Schmidt, R.; Kalman, A.; Chung, N. N.; Lemieux, C.; Horvath, C.; Schiller, P. W. Structure−activity relationships of dermorphin analogues containing N-substituted amino acids in the 2-position of the peptide sequence. *Int. J. Pept. Protein Res.* **1995**, *46*, 47−55.

(18) Manavalan, P.; Momany, F. A. Conformational energy studies on N-methylated analogues of thyrotropin releasing hormone, enkephalin, and luteinizing hormone-releasing hormone. *Biopolymers* **1980**, *19*, 1943−1973.

(19) Rajeswaran, W. G.; Hocart, S. J.; Murphy, W. A.; Taylor, J. E.; Coy, D. H. *N*-Methyl scan of somatostatin octapeptide agonists produces interesting effects on receptor subtype specificity. (in press, 2001.)

(20) 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.

(21) Hocart, S. J.; Jain, R.; Murphy, W. A.; Taylor, J. E.; Coy, D. H. Highly potent cyclic disulfide antagonists of somatostatin. *J. Med. Chem.* **1999**, *42*, 1863−1871.

(22) Shimon, Taylor, J. E.; Dong, J. Z.; Bitonte, R. A.; Kim, S.; Morgan, B.; Coy, D. H.; Culler, M. D.; Melmed, S. Somatostatin receptor subtype specificity in human fetal pituitary cultures − Differential role of SSTR2 and SSTR5 for growth hormone, thyroid-stimulating hormone, and prolactin regulation. *J. Clin. Invest.* **1997**, *99*, 789−798.

(23) Rossowski, W. J.; Coy, D. H. Specific inhibition of rat pancreatic insulin or glucagon release by receptor-selective somatostatin analogues. *Biochem. Biophys. Res. Commun.* **1994**, *205*, 341−346.

(24) Corletto, V. D.; Severi, C.; Coy, D. H.; Delle Fave, G.; Jensen, R. T. Colonic smooth muscle cells possess different subtypes of somatostatin receptor from gastric smooth muscle cells. *Am. J. Physiol.* **1997**, *272*, G689−G697.

(25) 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.

(26) Bruno, J.-F.; Xu, Y.; Song, J.; Berelowitz, M. Molecular cloning and functional expression of a novel brain specific SRIF receptor. *Proc. Nat. Acad. Sci. U.S.A.* **1992**, *89*, 11151−11155.

(27) 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.

(28) IUPAC−IUB Commission of biochemical nomenclature (CBN) symbols for amino acid derivatives and peptides, recommendations (1971). *Eur. J. Biochem.* **1972**, *27*, 201−207.

(29) 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.

(30) 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.

(31) 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.
(32) 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.
(33) 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.
(34) SYBYL molecular modelling software, version 6.6; Tripos Associates Inc.: 1699 Hanley Rd, St. Louis MO 63144-2913.
(35) Weiner, S. J.; Kollman, P. A.; Nguyen, D. T.; Case, D. A. An all atom force field for simulations of proteins and nucleic acids. *J. Comput. Chem.* **1986**, *7*, 230−252.
(36) McCammon, J. A.; Wolynes, P. G.; Karplus, M. Picosecond dynamics of tyrosine side chains in proteins. *Biochemistry* **1979**, *18*, 927−942.

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