N-Methyl Scan of Somatostatin Octapeptide Agonists Produces Interesting Effects on Receptor Subtype Specificity

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 analogues of somatostatin which exhibit selective affinities for the five receptor subtypes is of considerable basic and therapeutic interest and has generated a large number of potent agonist analogues with a wide spectrum of binding profiles. In the past, conformational restriction of side chain groups and the peptide backbone has yielded the most interesting results. Under the latter category and as part of the present study, we were interested in the potential effects of *N*-methylation of peptide bond NH groups on binding affinity since this approach had not been systematically examined with these peptides. This was aided by new chemistries for introducing an *N*-Me group during regular solid-phase peptide synthesis using Boc protection. A number of interesting effects were noted on relative binding affinities of the two series of agonist sequences chosen, DPhe[5](or Tyr[5])-*c*[Cys[6]-Phe[7]-DTrp[8]-Lys[9]-Thr[10]-Cys[11]]Thr[12]-NH_2 (SRIF numbering), at the five known human somatostatin receptors transfected into and stably expressed by CHO cells. *N*-Methylation of residues 7 (Phe), 10 (Thr), 11 (Cys), and 12 (Thr) largely destroyed affinities for all five receptors. *N*-Methylation of DTrp in the DPhe series gave an analogue with extraordinarily high affinity for the type 5 receptor for which it was also quite selective. *N*-Methylation of Lys in both series resulted in retention of type 2 affinity despite this residue constituting the "active center" of somatostatin peptides. *N*-Methylation of either the N-terminal Tyr residue or of Cys[6] in the Tyr series resulted in analogues with extraordinarily high affinity for the type 3 receptor, also with a degree of specificity. *N*-Methylation of the peptide bond constrains the conformational space of the amino acid and eliminates the possibility of donor hydrogen bond formation from the amide linkage. The β-bend conformation of the agonists around DTrp-Lys is stabilized by a transannular intramolecular hydrogen bond(s) between Phe[7] and Thr[10] so methylation of these residues eliminates this source of stabilization. It is expected that several of these analogues will provide additional tools for determining some of the physiological roles played by type 3 and 5 somatostatin receptors which are still far from being fully elucidated.

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, and 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 distributions 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 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 DTrp for LTrp, 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 series.[11] Thus, the many basic biological studies with this type of analogue failed to detect effects mediated by all but one of the SRIF receptor types. Since then, much work in our laboratory has gone into the reintroduction of broader-spectrum binding[12] into small, biologically stable peptides and also the development of peptides and peptidomimetics[13] with discrete specificity for a particular receptor.

One interesting approach, just made easier with new chemistry, was the introduction of a peptide backbone constraint imposed by *N*-alkylation. This could yield useful information about the bioactive conformation since *N*-methyl amino acid substitutions have often been used to increase the potency or selectivity of a peptide ligand.[14−17] This bulky steric modification blocks potential intramolecular hydrogen bonding sites and also proteolytic enzyme cleavage sites, thus potentially enhancing pharmacokinetic properties of a peptide. Only a few *N*-methyl amino acids are commercially available, and their synthesis is tedious.[13] In this paper, we have described *N*-methylation of truncated SRIF analogues

* 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 Analogue Structures and Analytical Data

			MS (M − H$^+$)		HPLC	
no.	code PRL-	*N*-Me sequence	calcd[a]	obsd[b]	t_{R-1}[c]	t_{R-2}[d]
1	3249	NMeDPhe-*c*[Cys-Phe-DTrp-Lys-Thr-Cys]-Thr-NH_2	1047.3	1047.7	12.6	12.7
2	3227	DPhe-*c*[NMeCys-Phe-DTrp-Lys-Thr-Cys]-Thr-NH_2	1047.3	1048.0	16.4	16.3
3	3259	DPhe-*c*[Cys-NMePhe-DTrp-Lys-Thr-Cys]-Thr-NH_2	1047.3	1048.8	14.8	14.4
4	3246	DPhe-*c*[Cys-Phe-NMeDTrp-Lys-Thr-Cys]-Thr-NH_2	1047.3	1047.3	17.3	16.6
5	3245	DPhe-*c*[Cys-Phe-DTrp-NMeLys-Thr-Cys]-Thr-NH_2	1047.3	1047.6	15.7	15.6
6	3250	DPhe-*c*[Cys-Phe-DTrp-Lys-NMeThr-Cys]-Thr-NH_2	1047.3	1047.8	9.4	9.4
7	3242	DPhe-*c*[Cys-Phe-DTrp-Lys-Thr-NMeCys]-Thr-NH_2	1047.3	1047.8	12.1	13.2
8	3226	DPhe-*c*[Cys-Phe-DTrp-Lys-Thr-Cys]-NMeThr-NH_2	1047.3	1048.1	10.8	10.9
9	1508	DPhe-*c*[Cys-Phe-DTrp-Lys-Thr-Cys]-Thr-NH_2	1033.2	1032.7	12.3	12.9
10	3128	NMeTyr-*c*[Cys-Phe-DTrp-Lys-Thr-Cys]-Thr-NH_2	1063.3	1063.8	13.7	13.4
11	3145	Tyr-*c*[NMeCys-Phe-DTrp-Lys-Thr-Cys]-Thr-NH_2	1063.3	1063.7	14.7	14.3
12	3144	Tyr-*c*[Cys-NMePhe-DTrp-Lys-Thr-Cys]-Thr-NH_2	1063.3	1063.4	13.4	13.6
13	3141	Tyr-*c*[Cys-Phe-NMeDTrp-Lys-Thr-Cys]-Thr-NH_2	1063.3	1063.7	15.9	15.8
14	3147	Tyr-*c*[Cys-Phe-DTrp-NMeLys-Thr-Cys]-Thr-NH_2	1063.3	1063.2	18.2	17.7
15	3185	Tyr-*c*[Cys-Phe-DTrp-Lys-NMeThr-Cys]-Thr-NH_2	1063.3	1063.0	11.7	11.5
16	3241	Tyr-*c*[Cys-Phe-DTrp-Lys-Thr-NMeCys]-Thr-NH_2	1063.3	1063.4	14.2	14.2
17	3157	Tyr-*c*[Cys-Phe-DTrp-Lys-Thr-Cys]-NMeThr-NH_2	1063.3	1063.7	11.9	11.6
18	3151	Tyr-*c*[Cys-Phe-DTrp-Lys-Thr-Cys]-Thr-NH_2	1049.2	1050.0	13.6	13.6

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

at every amino acid residue using the solid-phase procedure, adopted from a recent publication reported by Miller and Scanlan,[18] their binding affinity for the five cloned human SRIF receptors, and also their activity on inhibition of GH release from rat pituitary cells which is a subtype 2 receptor-mediated event.[6]

Results and Discussion

SRIF analogues **9** and **18** were alkylated[18] at every residue by a solid-phase procedure 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 NH of *o*-nitrobenzenesulfonamide was selectively deprotonated by the strong, hindered, nonionic base MTBD and methylated using methyl *p*-nitrobenzenesulfonate in DMF. The methylated sulfonamide was deprotected by β-mercaptoethanol and DBU in DMF, and this reaction was easily followed by the appearance of a bright yellow color in the solution, indicating the removal of the *o*-nitrobenzenesulfonyl group from the resin-bound peptide. Also, this deprotection is slower if the *N*-sulfonamide is not alkylated, thus capping the unalkylated peptide. The subsequent amino acid was coupled two times using TBTU/DIPEA instead of DIC.

Although this procedure appears to be amenable to most peptide sequences, one exception was identified during this research. The sequence (*o*-NBS)HN-Cys^{11}-(4-MeZ)-Thr^{12}(OBzl)-® could not be methylated using methyl *o*-nitrobenzenesulfonate, presumably due to steric hindrance created by the side chain protecting group(s). This problem was, however, circumvented by using dimethyl sulfate as a methylating agent instead of the bulky methyl *o*-nitrobenzenesulfonate.

The binding affinities (K_d, nM) of all SRIF analogues were determined using their concentration-dependent displacement of ^{125}I-radiolabeled peptide ligands from membranes isolated from CHO cells transfected with the corresponding human SRIF receptor 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 SRIF-14.[19] Given the profound effect which the conformation and side chain of the N-terminal amino acid have on the biological activities of this type of analogue,[11] two series of base structures (peptides **9** and **18**) were used for the present study − one containing a DPhe (analogue **9**) and the other a Tyr residue (analogue **18**) to give a total of 16 *N*-methylated analogues, the structures and physicochemical characteristics of which are given in Table 1.

Recent papers on the three-dimensional conformation of the SRIF agonist analogue Octreotide (DPhe-*c*[Cys-Phe-DTrp-Lys-Thr-Cys]-Thr-ol) described the X-ray crystallographic[20] and DMSO solution NMR conformations.[21] The compound was found to exist in three different conformations in the solid state with similar conformations for the sequence DPhe^5-Lys^9, an antiparallel β-sheet conformation characterized by a type II′ β-like turn spanning residues DTrp^8-Lys^9, stabilized by a Thr^{10}NH-Phe^{7}C═O hydrogen bond, and two folded structures in which the C-terminal tripeptide folded into a helical array stabilized by Cys^{11}NH-DTrp^{8}C═O and Phe^{7}NH-Thr^{10}C═O hydrogen bonds.[20] The NMR data demonstrated that a dynamic equilibrium exists between the relatively flat β-sheet form and the folded, partially helical conformations in solution.[21] The three NMR conformations of Octreotide were mutated to give models for the DPhe^5 analogues with a C-terminal amide group, based on the parent compound **9** (DPhe-*c*[Cys-Phe-DTrp-Lys-Thr-Cys]-Thr-NH_2). The addition of the bulky methyl group in place of the amide proton causes strain in many of the compounds, which is relieved by conformation changes. The extent of the conformation changes resulting from the addition of the sterically bulky methyl group can be readily demonstrated by the energy change that accompanies the conformational rearrangement. The Kollman all-atom energy differences for each conformation on sequential methylation

Table 2. Binding Affinities of Analogues Shown in Table 1 for Cloned Human sst_{1-5} Receptors and Agonist Activity on Culture Rat Pituitary Cells

peptide no.	N^{α}-methylation site	K_d[a] ± SEM (nM)					agonist IC_{50} ± SEM (n)[b] (nM)
		$hsst_1$	$hsst_2$	$hsst_3$	$hsst_4$	$hsst_5$	
SRIF-14	N/A[d]	2.0 ± 0.35	0.25 ± 0.03	1.2 ± 0.23	2.0 ± 0.25	1.4 ± 0.29	0.17 ± 0.054
SRIF-28	N/A[d]	1.9 ± 0.42	0.31 ± 0.06	1.3 ± 0.29	5.4 ± 2.5	0.4 ± 0.05	0.23 ± 0.052
1	DPhe^5	316 ± 11	1.03 ± 0.26	17.9 ± 2.5	>1000	4.89 ± 1.4	0.32 ± 0.13 (7)
2	Cys^6	378 ± 119	1.04 ± 0.18	13 ± 0.5	>1000	23.71	0.36 ± 0.19 (4)
3	Phe^7	>1000	13.17 ± 3.85	830 ± 86	>1000	83.24 ± 25.8	7.29 ± 2.08 (2)
4	DTrp^8	1200	23.5 ± 3.92	11.05 ± 1.03	>1000	0.61 ± 0.36	18.7 ± 8.1 (2)
5	Lys^9	867 ± 102	1.84 ± 0.21	67.48 ± 10.02	>1000	8.41 ± 6.85	0.74 ± 0.14 (4)
6	Thr^{10}	>1000	>1000	>1000	>1000	>1000	nd[c]
7	Cys^{11}	622 ± 172	56.23 ± 26.4	44.4 ± 8.36	574	28.42 ± 19.3	nd[c]
8	Thr^{12}	>1000	14.84 ± 1.53	124.3 ± 11.7	182	313	28.8 ± 8.0 (2)
9	N/A[d]	761	0.15 ± 0.08	11.84 ± 0.9	>1000	8.35	0.16 ± 0.04 (5)
10	Tyr^5	811 ± 188	9.74 ± 1.87	3.01 ± 1.05	nd[c]	27.00 ± 14.3	11.3 ± 2.5 (5)
11	Cys^6	862 ± 162	8.96 ± 1.66	2.73 ± 2.43	nd[c]	114.0	11.9 ± 4.1 (2)
12	Phe^7	653 ± 245	40.09 ± 3.79	94.20 ± 16.71	nd[c]	94.99 ± 22.0	103 ± 4.0 (2)
13	DTrp^8	1000	120.4 ± 22.2	8.00 ± 0.9	nd[c]	50.38 ± 28.6	nd[c]
14	Lys^9	956 ± 43	14.25 ± 3.12	51.02 ± 6.93	nd[c]	629 ± 371	27.4 ± 14.1 (2)
15	Thr^{10}	1000	61.35 ± 6.95	440 ± 126	1000	92.79 ± 0.7	nd[c]
16	Cys^{11}	1255	56.23 ± 26.4	17.00 ± 2.75	321	16.89	41.2 ± 31.9 (2)
17	Thr^{12}	611 ± 3.5	26.17 ± 10.3	535 ± 200	353	71.84 ± 15.5	nd[c]
18	N/A[d]	1000	10.33 ± 3.53	18.19 ± 4.21	nd[c]	32.95 ± 15.3	1.11 ± 0.07 (2)

[a] Expressed as the mean ± SEM; single values indicate the results of one binding experiment. [b] Rat in vitro agonist IC_{50} (nM) versus SRIF (1.0 nM), expressed as the mean ± SEM of n separate dose−response curves. [c] Not determined. [d] Not applicable.

Table 3. Kollman All-Atom Energy Change (kcal mol^{-1}) on Sequential Methylation of Each Residue of DPhe-*c*[Cys-Phe-DTrp-Lys-Thr-Cys]-Thr-NH_2 (**9**) in Each of the Three Solution Conformations

analogue no.	methylation Site	**I**	**II**	**III**
1	*N*MeDPhe^5	0.3	0.6	−0.5
2	*N*MeCys^6	6	9	7
3	*N*MePhe^7	12	37	28
4	*N*MeDTrp^8	4	7	5
5	*N*MeLys^9	6	7	7
6	*N*MeThr^{10}	20	15	19
7	*N*MeCys^{11}	10	21	15
8	*N*MeThr^{12}	12	28	30

are given in Table 3. These energy changes are driven by the steric bulk of the methyl group and are much larger than would be expected from the loss of a transannular hydrogen bond.

Analogue **9** displays very high affinity for type 2 receptors and high potency for inhibition of rat GH release but low affinity for the other receptor types which is typical for this class of peptide. Analogue **18** with its different N-terminal stereochemistry loses substantial affinity for all receptors tested. The DTrp-Lys sequence in these peptides is generally considered to be the active site motif, and few structural alterations are tolerated. We found, however, that N^{α}-methylation of DTrp in analogue **9** to give analogue **4** dramatically increased type 5 binding from 8.35 to 0.63 nM while decreasing affinity for sst_1, sst_2, and sst_4 receptors. This analogue thus has similar type 5 affinity to SRIF-28, and as far as we are aware, this is the most selective peptide type 5 ligand to be reported, although a quite selective peptidomimetic structure has also been reported.[13,22] The subtype 5 receptor has recently been implicated in mechanisms controlling GH and prolactin release from human pituitary cells,[23,24] which appear to behave markedly different from rat cells so that this analogue will prove to be a useful tool for evaluating solely type 5 receptor-mediated effects. *N*-Methylation of DTrp in analogue **18** to give analogue **13** decreased affinity for both the type 2 and 5 receptors, although type 3 affinity slightly increased. *N*-Methylation of Lys in the critical DTrp-Lys motif also yielded interesting results in that moderately high type 2 affinity and rat biological activity were retained in analogue **5** of the analogue **9** series, whereas most previous modifications to Lys have been poorly tolerated.

N-Methylation of the peptide bond constrains the conformational space of the amino acid and eliminates the possibility of donor hydrogen bond formation from the amide linkage. The β-bend conformation of the agonists around DTrp-Lys is stabilized by a transannular intramolecular hydrogen bond(s) between Phe^7 and Thr^{10} so methylation of these residues eliminates this source of stabilization.[20,21] However, the energy change is larger than would be expected from the loss of a hydrogen bond and is driven by the large steric bulk of the methyl group as evidenced by the large loss in energy relative to the unmethylated parents, **9** and **18**, for all three conformations (analogues **3** and **6**; see Table 3). The plane of the peptide bond between DTrp and Lys is roughly normal to the plane of the backbone ring so N^{α}-methylation of Lys^9 may be accommodated sterically without major disruptions to the conformation and the loss of energy on methylation is small with each conformation (<7 kcal mol^{-1}; see Table 3).

Other observations of particular note were modifications made to residues 5 and 6, particularly in the Tyr^5 (analogue **18)** series. *N*-Methylation of an N-terminal DPhe (analogue **1**) has been shown previously to retain high biological potency;[11] however, finding that *N*-methylation of residue 6 (Cys) to give analogue **2**, which retains most type 2 affinity and biological activity, is novel. Perhaps even more interesting were results obtained when the position 5 and 6 residues were methylated in the Tyr^5 (analogue **18)** series. Analogues *N*MeTyr^5 **(10)** and *N*MeCys^6 **(11)** had K_d's of 3.0 and 2.7 nM, respectively, for the type 3 receptor compared to 1.2 nM for SRIF-14 itself, and both analogues exhibited some increased selectivity for subtype 3, relative to the parent compound (**18**). However, the principal physiological functions of sst_3 still remain to be elucidated.

Methylation of the N-terminal exocyclic Tyr[5] or DPhe[5] residue increases the bulk of the N-terminal amine while changing its basicity and eliminating one possible hydrogen bond donor site. The experimental energy change is very small (**1**, <1 kcal mol^{-1}; see Table 3). Although position 6 is part of the cyclic peptide structure, the Cys[6]NH group is located outside the backbone ring and is externally oriented. Methylation thus has little affect on the ring conformation, and the observed energy change is smaller for the flat conformation **I**.

N^{α}-Methylation of positions 7, 10, 11, and 12 was detrimental to receptor affinity in both analogue series, with analogue **6** (*N*MeThr[10]) being almost devoid of affinity for all receptors. Residues 7 and 10 are involved in transannular hydrogen bonds, which stabilize the crucial β-bend around DTrp-Lys. To accommodate the large methyl group in *N*MePhe[7] (analogue **3**) or *N*MeThr[10] (**6**), the plane of the peptide bonds has to rotate markedly and a transannular hydrogen bond is eliminated. In each case, the energy change on methylation was large, with disruptions to the hydrogen bonding pattern and conformation of each of the three conformer models (≥10 kcal mol^{-1}; see Table 3). Interestingly, the energy change was smallest for the flat conformation **I**, with methylation of Phe[7], Cys[11], and Thr[12], whereas the energy change associated with the inactive analogue *N*MeThr[10] was smallest for conformation **II**. These data suggest that the conformational restrictions imposed by methylation of Thr[10] favor the helical conformation **II** over the flat conformation **I**. Similarly, methylation of residues 7, 11, and 12 favors the flat conformation **I** which may be the preferred conformation of the ligand in the binding site.

These results confirm 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 conformationally restricted peptides by N^{α}-alkylation and N^{α}-arylation is in progress, in this laboratory. Attempts will also be made to impose conformational restriction by cyclization using ring-closing metathesis techniques on solid phase.

Experimental Section

Abbreviations. The nomenclature for the SRIF 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.[27,28] Abbreviations of the common amino acids are in accordance with the recommendations of IUPAC−IUB.[29] Additional abbreviations: DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCM, dichloromethane; DIC, dicyclohexylcarbodiimide; DIEA, diisopropyethylamine; DMF, dimethylformamide; MTBD, 1,3,4,6,7,8-hexahydro-1-methyl-2*H*-pyrimido[1,2-*a*]pyrimidine; NPS, 2-nitrophenylsulfonyl; TBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid.

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 SRIF agonists 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 (3 washes); neutralization, 10% DIEA (1 min, 5 min); DMF wash cycle; DCM wash cycle (2 washes); double coupling; first with 1,3-diisopropylcarbodiimide esters (3 equiv), 30 min in DCM; DCM wash (3 washes); second coupling with pre-formed TBTU esters (3 equiv), 90 min in DMF, with a catalytic amount of DIEA; DMF wash (1 wash); DCM wash (3 washes). Coupling reactions were monitored qualitatively with the ninhydrin test.

***N*$^{\alpha}$-Protection.** After deblocking the amino group at the desired methylation site, the resin was suspended in DCM (20 mL). To this suspension were added collidine (3 equiv) and *o*-nitrobenzenesulfonyl chloride (3 equiv) 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 by qualitatively by the ninhydrin test.

***N*$^{\alpha}$-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 (4 washes).

***N*$^{\alpha}$-Me 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 (5 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 70% D at 1% min^{-1}. Column eluent was monitored at 215 nm. The retention time and purity of each peptide were assessed by the Rainin Dynamax HPLC Method Manager. Each peptide was found to have a purity of ≥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 hydrolysates following derivatization with *o*-phthaldialdehyde reagent (Sigma Chemical Co.) using an automatic HPLC system (Rainin Instrument Co.) fitted with a 100- × 4.6-mm, 3-μm C18 axial compression column with integral guard column (Microsorb AAAnalysis, type O;

Rainin Instrument Co.) The derivatized primary amino acids were eluted using a binary gradient of buffer A, 0.10 M sodium acetate containing 4.5% v/v methanol and 0.5% v/v tetrahydrofuran at pH 7.2, and buffer B, methanol. The gradient sequence: 0% A at 0 min, 35% A at 16.5 min, ,90% A at 30 min, and 90% A at 33 min was used with a flow rate of 1.0 mL min^{-1} at ambient temperature. Eluent was monitored at 340 nm and integrated by the Dynamax HPLC Method Manager (Rainin). Standard retention times were as follows: Asp, 6.6 min; Arg, 19.9 min; Trp, 25.4 min; 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.

SRIF Analogue Inhibition of GH Release. Anterior pituitaries from adult male rats were collected and dispersed by a previously described trypsin/DNase method.[30] 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 was tested in triplicate wells in a total volume of 1 mL medium 199 containing 1% BSA (fraction V; Sigma Chemical Co.). All wells contained GHRH-(1−29)NH_2 (1 nM). 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 media was measured by a standard double-antibody RIA using components generously supplied by the NHPP, NIDDK, NICHHD, and USDA. Agonist IC_{50}'s 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 SRIF Receptors. The genomic clones containing the human SRIF receptors ($hsst_{1-5}$)[27,31−34] were kindly provided by Dr. Graeme I. Bell (University of Chicago). The $hsst_1$, $hsst_2$, $hsst_3$, $hsst_4$, and $hsst_5$ cDNAs were isolated as a 1.5-kb *Pst*I-*Xmn*I fragment, 1.7-kb *Bam*HI-*Hin*dIII fragment, 2.0-kb *Nco*I-*Hin*dIII fragment, 1.4-kb *Nhe*I-*Nde*I fragment, and 1.2-kb *Hin*dIII-*Xba*I fragment, respectively, each containing the entire coding region of the full-length receptors. These fragments were independently subcloned into the corresponding restriction endonuclease sites in the mammalian expression vector pCMV5, downstream from the human cytomegalovirus (CMV) promoter, to produce the expression plasmids pCMV5/$hsst_1$, pCMV5/$hsst_2$, pCMV5/$hsst_3$, pCMV5/$hsst_4$, and pCMV5/$hsst_5$. For transfection into CHO-K1 cells, a plasmid, pRSV-neo (American Type Culture Collection, Rockville, MD), carrying the neomycin mammalian cell selectable marker was added.

Receptor Expression and Transfection. Transfections were performed by the calcium phosphate method. CHO-K1 cells were maintained in α-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} 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 cells 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, 3−5) 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; $hsst_5$, 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.

Molecular Modeling. All molecular modeling was performed on a Silicon Graphics Indigo^2 High Impact 10000 computer, using SYBYL 6.6[35] with the Kollman all-atom force field.[36] The PDB files for the three solution NMR structures of the initial compound Sandostatin/Octreotide, dPhe^5-*c*[Cys^6-Phe^7-D-Trp^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[37] 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. *Endocrine Rev.* **1995**, *16*, 427−442.
(5) Coy, D. H.; Taylor, J. E. Receptor-specific somatostatin analogues: correlations with biological activity. *Metabolism* **1996**, *34*, 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) 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.
(15) 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.
(16) Wormser, U.; Laufer, R.; Hart, Y.; Chorev, M.; Gilon, C.; Selinger, Z. Highly selective agonists for substance P receptor subtypes. *EMBO J.* **1986**, *5*, 2805−2808.
(17) Sugano, H. Synthesis and biological activity of peptides related to eledoisin. III. C-Terminal hexapeptide amides modified in methionine and isoleucine residues. *Bull. Chem. Soc. Jpn.* **1973**, *46*, 2168−2174 and references therein.
(18) Miller, S. C.; Scanlan, T. S. Site-selective n-methylation of peptides on solid support. *J. Am. Chem. Soc.* **1997**, *119*, 2301−2302.
(19) O'Carroll, A.-M.; Raynor, K.; Lolait, S. J.; and Reisine, T. Characterization of cloned human somatostatin receptor sstr5. *Mol. Pharmacol.* **1994**, *46*, 291−298.
(20) Pohl, E.; Heine, A.; Sheldrick, G.; Dautner, A.; Wilson, K. S.; Kallen, J.; Huber, W.; Pfaffli, P. J. Structure of octreotide, a somatostatin analogue. *Acta Crystallogr., Sect. D* **1995**, *51*, 48−59.
(21) Melacini, G.; Zhu, Q.; Goodman, M. Multiconformational NMR analysis of sandostatin (octreotide): Equilibrium between b-sheet and partially helical structures. *Biochemistry* **1997**, *36*, 1233−1241.
(22) Yang, L.; Guo, L.; Pasternak, A.; Mosley, R.; Rohrer, S.; Birzin, E.; Foor, F.; Cheng, K.; Schaeffer, J.; Patchett, A. A. Spiro[1H-indene-1,4′-piperidine] derivatives as potent and selective nonpeptide human somatostatin receptor subtype 2 (sst2) agonists. *J. Med. Chem.* **1998**, *41*, 2175−2179.
(23) Shimon, I.; 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.
(24) Jaquet, P.; Ouafik, L.; Saveanu, A.; Gunz, G.; Fina, F.; Dufour, H.; Culler, M. D.; Moreau, J. P.; Enjalbert, A. Quantitative and functional expression of somatostatin receptor subtypes in human prolactinomas. *J. Clin. Endocrinol. Metab.* **1999**, *84*, 3268−3276.
(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. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11151−11155.
(27) O'Carroll, A.-M.; Raynor, K.; Lolait, S. J.; and Reisine, T. Characterization of cloned human somatostatin receptor sstr5. *Mol. Pharmacol.* **1994**, *46*, 291−298.
(28) 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.
(29) IUPAC−IUB commission of biochemical nomenclature (cbn) symbols for amino acid derivatives and peptides, recommendations 1971. *Eur. J. Biochem.* **1972**, *27*, 201−207.
(30) Murphy, W. A.; Taylor, J.; Moreau, J.-P. and Coy, D. H. Novel heptapeptide somatostatin analogue displays antitumor activity independent of effects on growth hormone secretion. *Pept. Res.* **1989**, *2*, 128−132.
(31) Yamada, Y.; Post, S. R.; Wang, K.; Tager, H. S.; Bell, G. I. and 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.
(32) Yasuda, K.; Rens-Domiano, S.; Breder, C. D.; Law, S. F.; Sapel, C. B.; Reisine T. and Bell, G. I. Cloning of a novel somatostatin receptor, sstr-3, coupled to adenyl cyclase. *J. Biol. Chem.* **1992**, *267*, 20422−20428.
(33) Yamada, Y.; Reisine, T.; Law, S. F.; Ihara, Y.; Kubota, A.; Kagimoto, S.; Seino, M.; Seino, Y.; Bell, G. I. and 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.
(34) Rohrer, L.; Raulf, F.; Bruns, C.; Buettner, R.; Hofstaedter, F. and Schule, R. Cloning and characterization of a fourth human somatostatin receptor. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 4196−4200.
(35) SYBYL molecular modeling software, version 6.32, Tripos Associates Inc., 1699 Hanley Rd., St. Louis, MO 63144-2913.
(36) 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.
(37) 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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