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Articles

Design, Synthesis, and Biological Activities of Potent and Selective Somatostatin Analogues Incorporating Novel Peptoid Residues

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We report the synthesis, bioactivity, and structure−activity relationship studies of compounds related to the Merck cyclic hexapeptide c[Pro^6-Phe^7-D-Trp^8-Lys^9-Thr^{10}-Phe^{11}], L-363,301 (the numbering in the sequence refers to the position of the residues in native somatostatin). The Pro residue in this compound is replaced with arylalkyl peptoid residues. We present a novel approach utilizing β-methyl chiral substitutions to constrain the peptoid side-chain conformation. Our studies led to molecules which show potent binding and increased selectivity to the hsst2 receptor (weaker binding to the hsst3 and hsst5 receptors compared to L-363,301). In vivo, these peptoid analogues selectively inhibit the release of growth hormone but have no effect on the inhibition of insulin. The biological assays which include binding to five recombinant human somatostatin receptors carried out in two independent laboratories and in vivo inhibition of growth hormone and insulin provide insight into the relationship between structure and biological activity of somatostatin analogues. Our results have important implications for the study of other peptide hormones and neurotransmitters.

Introduction

Somatostatin, a tetradecapeptide hormone released by the hypothalamus, plays important physiological roles: it is a potent inhibitor of the release of several hormones (i.e., glucagon, growth hormone, insulin, gastrin) and regulates many other biological activities.[1] It is also highly active in acromegalic patients, lowering the plasma level of growth hormone, and therefore is of potential therapeutic value in clinical treatment of acromegaly and gastroenteropancreatic tumors.[2] Somatostatin induces its biological effects by interacting with a family of structurally related receptors. Five human somatostatin receptors have been cloned and referred to as hsst1−5 receptors.[3] The ligand SMS-201-995[4] (Octreotide, Sandostatin) and other clinically used somatostatin analogues interact with the receptor subtypes hsst2, -3, and -5 (we use the nomenclature suggested by Hoyer et al.).[5] The receptors hsst2 and -5 have been reported to mediate antiproliferative effects of somatostatin on tumor cell growth and therefore may mediate the clinical effects of Sandostatin in humans.[6]

The wide range of physiological roles of somatostatin against a number of endocrine hormones and its very short biological half-life have led to substantial efforts to synthesize peptide analogues in search of molecules exhibiting potent and selective biological activity and longer duration of action as compared to the hormone itself. In particular the studies of Veber and co-workers resulted in the discovery of the cyclic hexapeptide analogue c[Pro^6-Phe^7-D-Trp^8-Lys^9-Thr^{10}-Phe^{11}],[7] L-363,-301 (the numbering in the sequence refers to the position of the residues in native somatostatin). This molecule shows higher biological activity than native somatostatin in inhibiting the release of growth hormone, insulin, and glucagon. From NMR studies, Veber proposed a type II′ β-turn about the tetrapeptide sequence Phe^7-D-Trp^8-Lys^9-Thr^{10} which is considered the biological active portion, interacting with the receptor. The Phe^{11}-Pro^6 dipeptide is contained within a type VI β-turn which includes a cis peptide bond between the Phe^{11} and Pro residues. This dipeptide unit, the so-called bridging region, is important for maintaining the proper orientation of the tetrapeptide portion and contains a component of ligand−receptor interaction via the phenyl ring.[8] Hirschmann, Nicolaou, and their co-workers designed peptidomimetics of somatostatin employing a β-D-glucose scaffold.[9] They suggested that the peptide backbone was not required for receptor binding but served as a scaffold for supporting the side chains of Phe^7, Trp^8, Lys^9, and Phe^{11} in the required spatial arrangement. The contribution of the dipeptide Phe^{11}-Pro^6 to the binding affinities of somatostatin analogues has been investigated thoroughly.[10,11]

We have previously carried out extensive studies on the structure−activity relationships of somatostatin analogues related to L-363,301 in which the side-chain torsions were constrained as a result of C$^\beta$-methylation.[12,13] Results obtained from the conformational

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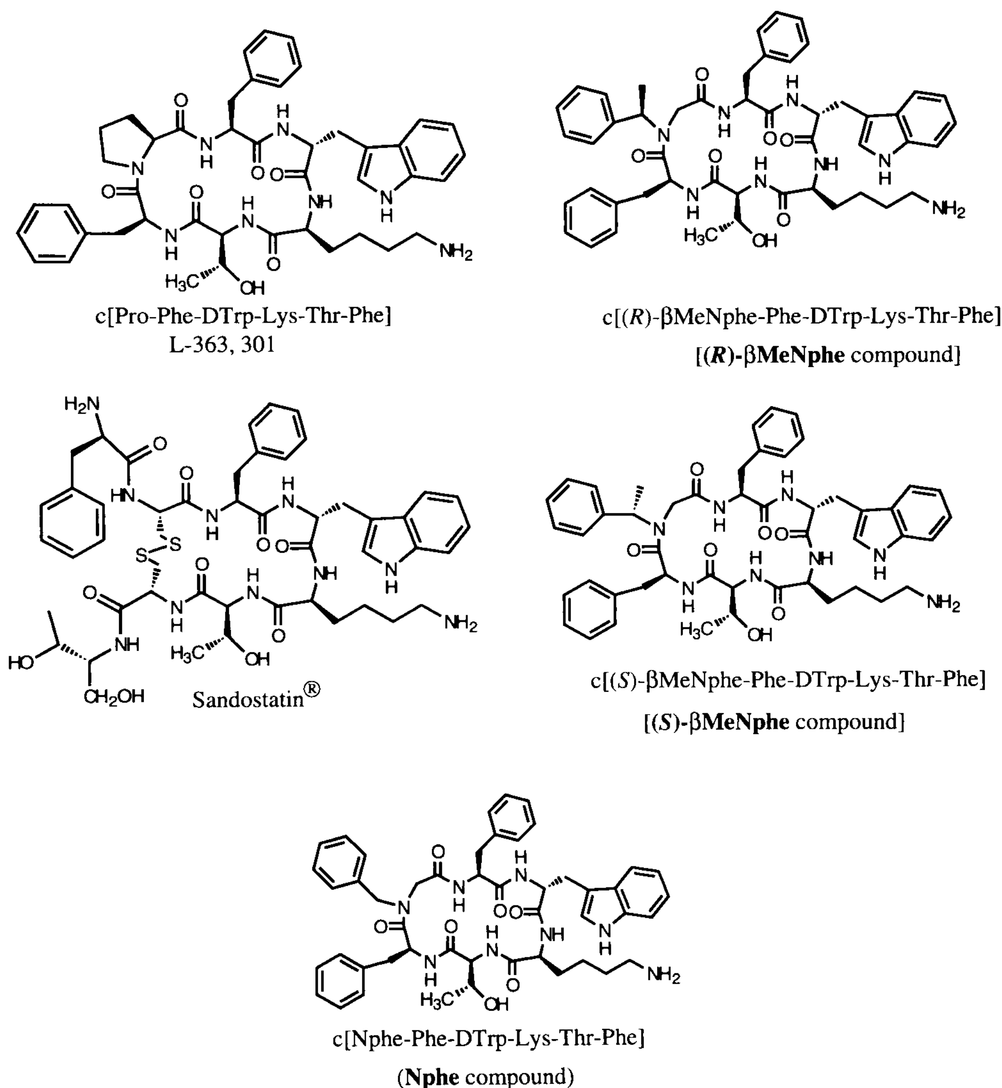
Chart 1. Structures of L-363,301, Sandostatin, NPhe, (*R*)-*β*MeNphe, and (*S*)-*β*MeNphe Analogues



analysis of these analogues revealed the features of a side-chain topology of analogues that bind well to somatostatin receptors.

Design Rationale

In our current studies we investigate the role of the bridging region by incorporating novel peptoid residues. Peptoids represent a new class of imino acid residues that are not found in nature and have been shown to possess significant proteolytic stability.[14]

Our studies of somatostatin analogues are based on the Merck compound c[Pro^6-Phe^7-D-Trp^8-Lys^9-Thr^{10}-Phe^{11}], L-363,301. An arylalkyl peptoid residue, *N*-benzylglycine ("Nphe"), is incorporated into position 6 of L-363,301 resulting in c[Nphe^6-Phe^7-D-Trp^8-Lys^9-Thr^{10}-Phe^{11}] **(Nphe compound)** (Chart 1). The rationale of this design is based on several considerations. Peptoid structures can induce cis amide bonds as does Pro^6 in L-363,301. The functional groups in the peptoid residue can be readily altered, and therefore structural rigidity, complexity, and diversity of the designed analogues can be increased easily. NMR studies on L-363,301 have established that in solution the phenyl ring of Phe^{11} is positioned close to the proline ring.[10] In addition, a model developed in our group has shown that the aromatic side chain of the D-Phe residue of Sandostatin is in spatial proximity of the bridging region.[15] We envisioned that the incorporation of a peptoid residue containing an aromatic side chain into position 6 can occupy some of the conformational space available to Phe^{11} in L-363,301 and/or the D-Phe in Sandostatin.

We made further attempts to constrain the peptoid side chain by incorporation of novel chiral *β*-methylated peptoid residues. Two side-chain-methylated peptoid residues, (*R*)- and (*S*)-N(αMe)benzylglycines [(*R*)- and (*S*)-*β*MeNphe], were designed and incorporated into position 6 to give the two analogues c[(*R*)-*β*MeNphe^6-Phe^7-D-Trp^8-Lys^9-Thr^{10}-Phe^{11}] **[(*R*)-*β*MeNphe** compound] and c[(*S*-*β*MeNphe^6-Phe^7-D-Trp^8-Lys^9-Thr^{10}-Phe^{11}] **[(*S*)-*β*MeNphe** compound]. The conformational analysis of our peptoid analogues using ^{1}H NMR and computer simulations is described in the accompanying paper.[17]

Synthesis

The synthesis of the peptoid residues was achieved by alkylation of benzyl-, (*R*)-α-methylbenzyl-, or (*S*)-α-methylbenzylamines with ethyl bromoacetate.[18] The routes of synthesis of the peptoid analogues are shown in Scheme 1. The syntheses of these three analogues

Scheme 1. Synthesis of c[(*R*)-βMeNphe-Phe-D-Trp-Lys-Thr-Phe]

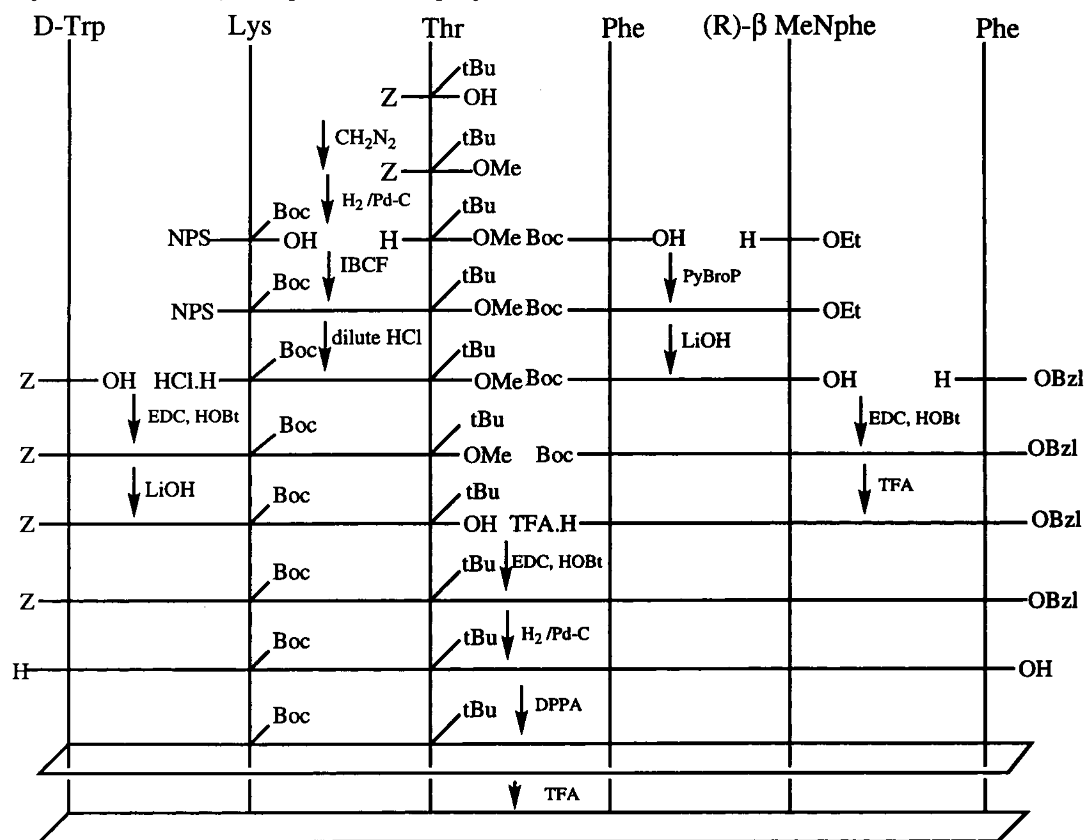


Table 1. In Vitro Inhibition of Radioligand Binding to Human Recombinant Receptors: K_i (nM) ± SEM[a,b]

compound	hsst1	hsst2	hsst3	hsst4	hsst5	hsst5/hsst2	hsst3/hsst2
L-363,301	>1000	5.10 ± 0.76	129 ± 51	>1000	25.0 ± 6.8	4.9	25.3
Phe-Nphe	>1000	6.98 ± 0.83	253 ± 57	>1000	100.7 ± 45.5	14.4	36.2
Phe-(*R*)-βMeNphe	>1000	2.33 ± 0.41	425 ± 100	>1000	33.5 ± 12.5	14.4	182.4
Phe-(*S*)-βMeNphe	>1000	29.5 ± 2.49	797 ± 125	987 ± 13	87.0 ± 22.6	2.9	27.0

[a] Data obtained from John Taylor. [b] Binding assays were carried out with cell membranes from CHO-K1 cells.

were designed around the reaction between the tripeptide Cbz-D-Trp-Lys(Boc)-Thr(tBu)-OH[19] and the other tripeptide Boc-Phe-Nxaa-Phe-OBzl (Nxaa = Nphe, (*R*)- or (*S*)-βMeNphe) fragments incorporating the bridging region. The Boc-Phe-Nxaa-Phe-OBzl tripeptides were synthesized in solution in a stepwise manner with benzyl and ethyl ester protection for the C-terminals and Boc protection for the α-amino groups. Ethyl[(*N,N*-dimethylamino)propyl]carbodiimide (EDC) was used as coupling agent in the presence of hydroxybenzotriazole (HOBt) to prepare the protected tripeptides. For the more hindered coupling of Boc-Phe-OH with (*R*)- or (*S*)-βMeNphe-OEt, the highly efficient coupling reagent PyBroP was used instead of EDC/HOBt.

The fully protected linear hexapeptides were obtained from fragment condensation reactions between Cbz-D-Trp-Lys(Boc)-Thr(tBu)-OH and H-Phe-NXaa-Phe-OBzl using EDC/HOBt. Upon reductive removal of the benzyloxycarbonyl and benzyl ester protections, the deprotected linear hexapeptides were allowed to cyclize at 1 mM concentration using DPPA/K_2HPO_4. Simultaneous acidolysis of *tert*-butyl ether and N^{ϵ}-Boc protections from the protected cyclic compounds was carried out with TFA in the presence of scavengers to give the desired cyclic hexapeptides, which were purified by reversed-phased HPLC. Detailed synthesis and characterization of all new compounds are given in the Supporting Information.

Biological Results and Discussion

The somatostatin analogues were tested in vitro for their specific binding to the five human somatostatin receptors expressed in CHO cell lines (Table 1, hsst1−5 receptors; Table 2, hsst2−4 receptors) hSF9 cells (Table 2, hsst1) or CCL-39 cell lines (Table 2, hsst5 receptor) and in vivo for their ability to inhibit the release of growth hormone and insulin in rats. The in vitro tests were carried out in two independent laboratories, J.T. (Table 1) and D.H. (Table 2). The binding potencies of the peptoid analogues and the ratios of the binding potencies to the hsst5 and hsst2 as well as the ratios of the binding potencies to the hsst3 and hsst2 are given in Tables 1 and 2. Both sets of binding constants show that the Nphe and the (*R*)-βMeNphe compounds bind more effectively than the (*S*)-βMeNphe to the hsst2 receptor. The binding constants for the hsst2 receptor obtained by J.T. suggest that the (*S*)-βMeNphe binds 1 order of magnitude weaker than the (*R*)-βMeNphe and 4 times weaker than the Nphe compound, while the data obtained by D.H. show that the Nphe and the (*R*)-βMeNphe compounds are equipotent and 3-fold more active in the hsst2 receptor compared to the (*S*)-

Table 2. In Vitro Inhibition of Radioligand Binding to Human Recombinant Receptors: K_d (nM) ± SEM[a,b]

compound	hsst1	hsst2	hsst3	hsst4	hsst5	hsst5/hsst2	hsst3/hsst2
L-363,301	4570 ± 106	0.76 ± 0.05	525 ± 37	8913 ± 420	7.6 ± 1.5	10	690
Phe-Nphe	7080 ± 333	2.95 ± 0.71	282 ± 27	4680 ± 210	69 ± 3	23.4	158.6
Phe-(*R*)-βMeNphe	3550 ± 431	2.04 ± 0.75	407 ± 50	4168 ± 195	288 ± 7	141.2	199.5
Phe-(*S*)-βMeNphe	13183 ± 1254	6.17 ± 1.34	339 ± 68	2630 ± 460	115 ± 3	18.6	54.9

[a] Data obtained from Daniel Hoyer. [b] Binding assays were carried out with cell membranes from CHO cells for hsst2−4 receptors, hSF9 cells for the hsst1 receptor, and CCL-39 cells for the hsst5 receptor.

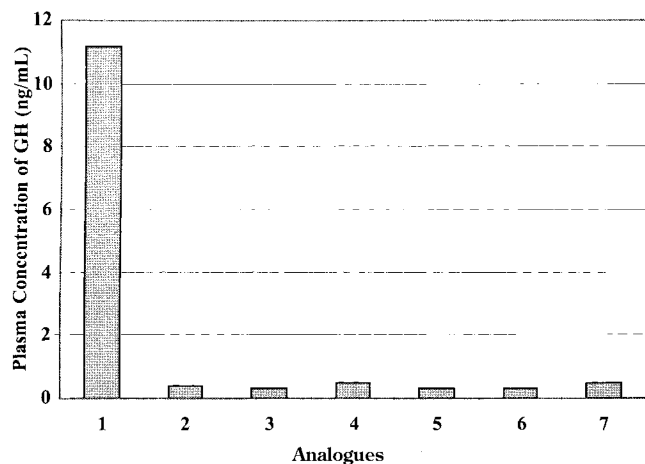


Figure 1. Effect of somatostatin analogues on GH release in rats: **1**, control, $n = 20$; **2**, Sandostatin, 100 μg/kg, $n = 24$; **3**, RC-160 (H-D-Phe-c[Cys-Tyr-D-Trp-Lys-Val-Cys]-Trp-NH_2), 100 μg/kg, $n = 10$; **4**, L-363,301, 100 μg/kg, $n = 10$; **5**, Nphe analogue, 100 μg/kg, $n = 15$; **6**, (*R*)-βMeNphe analogue, 100 μg/kg, $n = 15$; **7**, (*S*)-βMeNphe analogue, 100 μg/kg, $n = 10$.

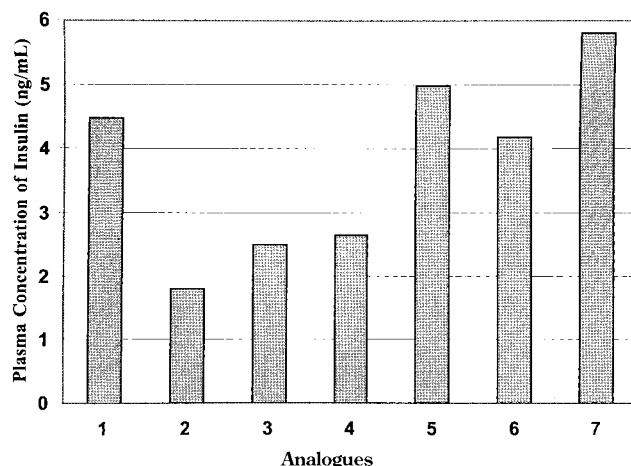


Figure 2. Effect of somatostatin analogues on insulin release in rats: **1**, control, $n = 15$; **2**, Sandostatin, 100 μg/kg, $n = 10$; **3**, RC-160 (H-D-Phe-c[Cys-Tyr-D-Trp-Lys-Val-Cys]-Trp-NH_2), 100 μg/kg, $n = 10$; **4**, L-363,301, 100 μg/kg, $n = 10$; **5**, Nphe analogue, 100 μg/kg, $n = 10$; **6**, (*R*)-βMeNphe analogue, 100 μg/kg, $n = 10$; **7**, (*S*)-βMeNphe analogue, 100 μg/kg, $n = 10$.

βMeNphe. The results obtained for the hsst5 receptor indicate considerable variations in binding constants. The data obtained by J.T. show that the Nphe and the (*S*)-βMeNphe have reduced potencies at the hsst5 receptor compared to L-363,301, while the (*R*)-βMeNphe compound is insignificantly less potent in the hsst5 compared to L-363,301. The binding constants measured by D.H., on the other hand, suggest a reduced potency at the hsst5 receptor for the (*R*)-βMeNphe compared to the Nphe and (*S*)-βMeNphe. Since the two laboratories use different cell lines for the hsst5 receptor and different radioligands as well as slightly different protocols (see the Experimental Section), it is difficult to compare the two results in quantitative terms.

In qualitative terms the results obtained from both laboratories are consistent at the hsst3 receptor. All three peptoid analogues show reduced potency at the hsst3 receptor compared to L-363,301. Both sets of binding data indicate that the peptoid analogues, especially the Nphe and (*R*)-βMeNphe, are hsst2-selective somatostatin analogues.

The changes in binding suggest critical topochemical requirements for the peptoid side chains. Spatial proximities between the three aromatic side chains of Phe^{11}, $Nxaa,^{6}$ and Phe^{7} appear to influence the selectivity and binding potency of these compounds. It is reasonable to assume that aromatic stacking in positions 11, 6, and 7 of the cyclic hexapeptides plays an important role in stabilizing the active conformations of these peptides. Indeed, Veber proposed a hydrophobic stacking of Phe^{11}-Pro^{6}-Phe^{7} side chains based on marked upfield shifts in the proton NMR spectrum.[8]

The effects of the peptoid analogues of somatostatin on the release of insulin and growth hormone were examined in vivo in rats. Sandostatin, RC-160 (H-D-Phe-c[Cys-Tyr-D-Trp-Lys-Val-Cys]-Trp-NH_2), and L-363,301 served as reference compounds. Figure 1 shows that the peptoid analogues Nphe, (*R*)-βMeNphe, and (*S*)-βMeNphe are as active as Sandostatin, RC-160, and L-363,301 in the inhibition of growth hormone. These results are in good agreement with the hypothesis that hsst2 receptor is responsible for the inhibition of the GH release.[5,20]

Figure 2 demonstrates that analogues Nphe, (*R*)-βMeNphe, and (*S*)-βMeNphe have no effect on the inhibition of insulin compared to Sandostatin, RC-160, and L-363,301 under these experimental conditions. The statistical evaluation and results of tests at different concentrations are given in Figures 1S−3S in the Supporting Information. The incorporation of the arylalkyl peptoid residues results in a loss of inhibition of insulin release while these analogues remain active in the inhibition of GH release. This observation could suggest that the phenyl ring of the peptoid residue at position 6 may interact directly with the receptor responsible for the GH release while it prevents interaction with the receptor associated with insulin release. Detailed understanding of these changes could prove valuable for the design of functionally selective analogues.

Conclusions

Modifications in the bridging region resulted in somatostatin analogues that are selective in the in vitro binding to the human somatostatin receptor subtype hsst2. Peptoid residues were designed which effectively replace the proline of L-363,301 and have led to the

preparation of somatostatin analogues with modified biological activities with respect to binding to isolated receptors and inhibition of GH and insulin release. A structure–activity relationship study was carried out in order to optimize the side-chain orientation of the analogues, and the (*R*)-βMeNphe analogue was found to be the most potent compound in this series with regard to the binding to the hsst2 receptor. Indeed, the position 6 in L-363,301 peptide could be the most interesting position in terms of alteration of binding affinity. These studies clearly demonstrate a novel approach using side-chain-methylated peptoids to elucidate bioactive conformations of somatostatin analogues.

The finding that injections of our analogues into rats did not influence insulin secretion indicates that under these given experimental conditions the peptoid-containing compounds might selectively inhibit the GH compared with Sandostatin, RC-160, and L-363,301 which are very potent in the inhibition of both GH and insulin.[7] The peptoid analogues inhibit the secretion of growth hormone in doses comparable to that of RC-160, L363,301, and Sandostatin. While RC-160, L-363,-301, and Sandostatin inhibit the release of insulin at these doses (100 μg/kg), the peptoid analogues have no effect on insulin secretion. This group of new target molecules should be very useful for delineating the roles played by the somatostatin receptors in mediating the variety of important physiological processes under the inhibitory control of somatostatin. The fact that relatively simple modifications of biologically active peptides led to potent and selective analogues opens new exciting opportunities in drug design. These results are fully consistent with the results of our receptor-based approach to drug design.[21] These analogues will be an important guide in efforts to design therapeutically useful somatostatin analogues.

Experimental Section

Abbreviations used are those recomended by the IUPAC–IUB Commission: Bzl, benzyl; Boc, (*tert*-butyloxy)carbonyl; tBu, *tert*-butyl; Cbz, benzyloxycarbonyl; DPPA, diphenyl phosphorazidate; EDC, 1-ethyl-3-[3′-(dimethylamino)propyl]carbodiimide; GH, growth hormone; HOBt, 1-hydroxybenzotriazole; HPLC, high-pressure liquid chromatography; PyBroP, bromotris(pyrrolidino)phosphonium hexafluorophosphate; hsst receptor, human somatostatin receptor; TFA, trifluoroacetic acid. [^{125}I]CGP 23996 (c[Lys-Asu-Phe-Phe-Trp-Lys-Thr-(^{125}I-Tyr)-Thr-Ser]), [^{125}I]Tyr^3-octreotide (D-Phe-c[Cys-(^{125}I-Tyr)-D-Trp-Lys-Thr-Cys]-Thr-OH), [^{125}I]Leu^8, D-Trp^{22}, and Tyr^{25}(LTT)-SRIF$_{28}$ (Ser-Ala-Asn-Ser-Asn-Pro-Ala-Leu-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-c[Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-(^{125}I-Tyr)-Thr-Ser-Cys]-OH) were purchased from ANAWA AG (Wangen, Switzerland).

Receptor Binding Assays. 1. J.T. (Table 1). Stable Expression of hsst Receptor Subtypes. The complete coding sequences of genomic fragments of the hsst1–4 receptor genes and a cDNA clone for the hsst5 were subcloned into mammalian expression vector pCMV. Clonal cell lines stably expressing the hsst1–5 receptors were obtained by transfection into CHO-K1 cells (ATCC) using the calcium phosphate coprecipitation method.[22] The plasmid pRSV-neo (ATCC) was included as a selectable marker. Clonal cell lines were selected in RPMI 1640 media containing 0.5 mg/mL G418 (Gibco), ring-cloned, and expanded into culture.

Radioligand Binding Assays. Membranes for in vitro receptor binding assays were obtained by homogenizing (Polytron setting 6, 15 s) the CHO-K1 cells, expressing the hsst receptor subtypes, in ice-cold 50 mM Tris-HCl and centrifuging twice at 39000*g* (10 min), with an intermediate resuspension in fresh buffer. The final pellets were resuspended in 10 mM Tris-HCl for assay. For the hsst1,3,4,5 assays, aliquots of the membrane preparations were incubated (30 min/37 °C) with 0.05 nM [^{125}I-Tyr^{11}]SRIF-14 in 50 mM HEPES (pH 7.4) containing BSA (10 mG/mL), $MgCl_2$ (5 mM), Trasylol (200 kIU/mL), bacitracin (0.02 mg/mL), and phenylmethanesulfonyl fluoride (0.02 mg/mL). The final assay volume was 0.3 mL. For the hsst2 assay, [^{125}I]MK-678 (0.05 nM) was employed as the radioligand and the incubation time was 90 min/25 °C. The incubations were terminated by rapid filtration through GF/C filters (presoaked in 0.3% poly(ethylenimine)) using a Brandel filtration manifold. Each tube and filter were then washed three times with 5-mL aliquots of ice-cold buffer. Specific binding was defined as the total radioligand bound minus that bound in the presence of 1000 nM SRIF-14 (hsst1,3,4,5) or 1000 nM MK-678 (hsst2).

2. D.H. (Table 2). Cell culture and expression of human SRIF receptors in CHO or CCL39 cells were as reported previously.[23] Insect SF9 cells expressing hsst1 receptors were obtained as pellets. Cells were washed twice with 10 mmol of HEPES (pH 7.6) and harvested and membranes prepared as described.[24] Membranes were resuspended into 10 mmol/L HEPES (pH 7.6) containing 5 mmol/L $MgCl_2$, 10 mg/mL bacitracin, and 0.5% (w/v) bovine serum albumin (BSA).

For crude membrane preparations, cells were harvested by washing with 10 mM HEPES, pH 7.5, scrapping off the culture plates with 4 mL of the same buffer, and centrifugation at 4 °C for 5 min at 2500*g*. The cell pellet was either stored at −80 °C or directly used. The cells were resuspended in binding assay buffer (10 mM HEPES, pH 7.5, 0.5% (w/v) BSA) by homogenization with the polytron at 50 Hz for 20 s.

Radioligand Binding Assay. Following radioligands were used for human SRIF receptor binding: [^{125}I]LTT-SRIF$_{28}$ (hsst1 SF9, hsst3 CHO, hsst5 CCL-39), [^{125}I]CGP 23996 (hsst4, CHO), and [^{125}I]Tyr^3-octreotide (hsst2, CHO).

In competition experiments, 150 μL of the cell or membrane homogenate was incubated with 50 μL of [^{125}I]LTT-SRIF$_{28}$, [^{125}I]SRIF$_{14}$, [^{125}I]CGP 23996, or [^{125}I]Tyr^3-octreotide (2175 Ci/mmol, 25–35 pM, final concentration each), respectively, in binding assay buffer containing $MgCl_2$ (5 mM), the protease inhibitor bacitracin (5μg/mL), and either 50 μL of binding assay buffer (total binding) or 50 μL of various peptide concentrations. Nonspecific binding was determined in the presence of 50 μg of SRIF$_{14}$ (1 μM). After 1 h at room temperature, the incubation was terminated by vacuum filtration through glass fiber filters presoaked in 0.3% (w/v) poly(ethylenimine). The filters were rinsed twice with 5 mL of ice-cold 10 mM Tris/HCl buffer, pH 7.4, and dried. Bound radioactivity was measured in a γ-counter (80% counting efficiency).

Data Analysis. Competition and saturation curves from experiments performed in triplicate determination were analyzed as described previously.[25] Data were analyzed by nonlinear regression curve fitting with the computer program SCTFIT.[26] The data are reported as pK_D values (−log mol/L). Protein concentrations were determined by the method of Bradford[27] with bovine serum albumin as a standard.

Animal Studies: Growth Hormone and Insulin Tests. Animals and Treatment. In vivo studies were perfomed with Wistar male rats (Harlan, Israel), weighing 180–220 g. Animals were maintained as outlined in the *Guide for the Care and Use of Laboratory Animals* (NIH Publication no. 85-23, 1985). The animals were allowed free access to food and water until 18 h before the experiment, when all the food (but not the water) was withdrawn. All animals were housed in cages with wide mesh wire bottom to prevent coprophagia (feeding on excrement).

Experiments were carried out under Nembutal anesthesia (60 mg/kg ip), to stimulate GH release (at time 0). All drugs were dissolved in saline (0.9% NaCl) and administrated sc (10 min following anesthesia), at a final dose of 100 μg/kg. The control group received 1 mL/kg saline (0.9% NaCl). At 25 min femoral cannulation was performed, and 0.5 g/kg L-Arg (dis-

solved in saline) was administrated intravenous in order to stimulate insulin release. The "none" group did not receive L-Arg stimulation. Blood was collected 5 min following L-Arg stimulation from the inferior vena cava for GH and insulin measurements. Plasma heparin (15 U/mL) was obtained from blood samples and stored at −20 °C until assay.

Hormone Assays. Rat growth hormone (rGH) ^{125}I levels were determined by appropiate Amersham's assay system. The standard in this kit has been calibrated against a reference standard preparation (NIH-RP2) obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). Rat insulin (rInsulin) ^{125}I levels were measured by appropiate Linco's assay system.

All samples were measured by duplicate. All samples were diluted 1:1 with assay buffer. Plasma concentrations were calculated from standard curve after consideration of the dilution factor.

Statistical Methods. Plasma hormone concentrations were expressed as means and standard error of the means (mean ± SEM). One-way ANOVA followed by Fisher's contrast was used to compare between groups. A P value of <0.05 was considered statistically significant. Each group consisted of $n = 10-12$ subjects; animals were divided randomly between groups.

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Supporting Information Available: Complete experimental descriptions for synthesis of Nphe, (*R*)-βMeNphe, and (*S*)-βMeNphe analogues and supplementary Scheme 1 for the preparation of Nphe compound. (18 pages). Ordering information can be found on any current masthead page.

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