# SYNTHESIS AND BIOLOGICAL ACTIVITY OF HIGHLY POTENT HEPTAPEPTIDE ANALOGS OF SOMATOSTATIN WITH C-TERMINAL MODIFICATIONS

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**Objective**; Structure-activity relationship studies of a series of new heptapeptide analogs of somatostatin with C-terminal modifications were performed.

**Methods;** New somatostatin analogs were synthesized by solid-phase method. The suppression of the release of growth hormone by the new analogs was assayed *in vivo* in rats, and the duration of action was investigated using growth hormone time-course assay. Growth hormone concentration in plasma samples was determined by RIA.

**Results**; The growth hormone release inhibitory activity of the new analogs was up to 50 times greater than that of somatostatin. Hydrophilic substitutions at the C-terminus seemed to be advantageous for potency. The most potent analog of the series, D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-NH-$CH_2$-$CH_2$-OH showed the most prolonged activity by inhibiting the release of growth hormone for at least 3 h. The analogs containing D-Phe or D-Tyr at the N-terminus were almost equipotent, which proves that the exocyclic N-terminal residue is not involved directly in the receptor recognition.

**Conclusions;** New heptapeptide analogs of somatostatin with C-terminal modifications were more potent and longer acting than the native hormone.

**Key words**: Somatostatin analogs – Solid phase peptide synthesis – Structure-activity relationships

The peptide hormone somatostatin (SRIF), a cyclic tetradecapeptide whose primary structural formula is:

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 1   2   3   4   5   6   7   8
Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp
          |                   |
         Cys-Ser-Thr-Phe-Thr-Lys
         14  13  12  11  10   9
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was isolated by BRAZEAU et al. from ovine hypothalami in 1973.

Somatostatin, first known as an inhibitor of the release of growth hormone (GH), was soon found to have an inhibitory action on a variety of physiological functions in hypothalamus, the anterior pituitary gland, the gastrointestinal tract and pancreas (GUILLEMIN and GERICH 1976). In addition, because of its distribution in the various brain regions and spinal cord, it has been suggested that somatostatin plays a role in neural transmission (EPELBAUM 1986).

Somatostatin induces its biological effects, all inhibitory in nature, by interacting with a family of structurally related, G protein-coupled, transmembrane receptors, located in the pituitary, the pancreas, the gastrointestinal tract, and the brain, as well as in various tumor cells. Five human somatostatin receptors, have been cloned and character-

ized (YAMADA et al. 1992, 1993). Their specific functional roles are under extensive investigation.

Because somatostatin has a wide range of physiological functions and possible clinical applications, extensive studies have been carried out to investigate its structure-activity relationships. The most possible conformation of the cyclic part of somatostatin, proposed by FREIDINGER et al. (1984), is that of type II' ß-turn about the tetrapeptide sequence Phe$^{7}$-Trp$^{8}$-Lys$^{9}$-Thr$^{10}$, which is considered the biologically active portion, interacting with the receptor.

Since the discovery of somatostatin much effort has been paid to ascertain the minimum structural requirements for biological activity. Initial work in the development of somatostatin analogs was done by VALE et al. (1977). RIVIER et al. (1975) found that replacing of Trp$^{8}$ by D-Trp$^{8}$ increased potency 6-8 times (inhibition of GH *in vitro* and glucagon and insulin *in vivo*). [D-Trp$^{8}$]SRIF was the first analog to be reported to have significantly higher potency than somatostatin.. In view of our present knowledge D-Trp$^{8}$ substitution seems to be critical for potency of somatostatin analogs, since it has been used in all SRIF analogs thereafter**.**

A breakthrough in somatostatin analogs design was made by BAUER et al. (1982) who synthesized an octapeptide analog, called SMS 201-995 or octreotide, of the following sequence:

D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol
| |
S————————————S

Octreotide was characterized by greater potency than the native somatostatin, longer duration of action (half life of octreotide is approximately 117 min.), greater metabolic stability and higher selectivity in GH inhibition as compared with insulin and glucagon. Since its discovery hundreds of octreotide analogs have been synthesized in search for even more potent compounds.

In this paper we report the synthesis and biological activity of several new heptapeptide somatostatin analogs based on octreotide with C-terminal modifications.

## Materials and Methods

**Starting materials.** The amino acid derivatives: Boc-Phe, Boc-D-Phe, Boc-D-Tyr(2-Br-Z), Boc-Cys (4-MeBzl), Boc-D-Trp, Boc-Lys(2-Cl-Z), Boc-Thr(Bzl), and Boc-Cys(4-MeBzl)-Merrifield resin were purchased from Chem Impex Int. Isopropylamine, 2-amino-2-methyl-1,3-propandiol, ethanoloamine, and ethylenediamine were purchased from Aldrich, and TBTU (2-[1H-benzotriazol-1-yl]-1,1,3,3-tetramethyluronium tetrafluoroborate) from Bachem.

**Peptide synthesis.** The analogs were assembled manually, using standard solid-phase procedures (MERRIFIELD, 1963) on Boc-Cys(4-MeBzl)-Merrifield resin (0.4 mequiv g$^{-1}$) in 0.25 mmol scale using *tert*-butyloxycarbonyl (Boc) group for N$^{\alpha}$-amino protection and TBTU as a condensing reagent. The coupling time was 180 min. A 3-fold excess of protected amino acids was used.

Heptapeptides were cleaved from the resin support with 50% solution of appropriate amine : (2-amino-2-methyl-1,3-propandiol, isopropylamine, ethanoloamine or ethylenediamine) in methanol, followed by removing all protective groups with anhydrous hydrogen fluoride (HF), containing the scavengers: anisole and dithiotreitol, for 60 min. at 0$^{o}$C. The peptides were cyclized in 90 % acetic acid (500 ml) with a slight excess of $I_2$ (15 min.). Excess $I_2$ was then removed by the addition of ascorbic acid (HOCART et al., 1999).

After cyclization, disulfide peptides were subjected to gel filtration on a 3 x 110 cm Sephadex G-10 column in 5 % AcOH, followed by chromatography on Sephadex LH-20, in the solvent system $H_2O$:n-BuOH:$CH_3COOH$:MeOH 90:10:10:8. The purity of the final products was checked by analytical HPLC, on a $C_{18}$ column eluted with solvent A (0.1% aqueous $CF_3COOH$) and solvent B (0.1 % $CF_3COOH$ in 80 % $CH_3CN$). The purified peptides were characterized by FAB-MS and amino acid analysis.

**GH potency assay**. Adult male Long-Evans rats weighing 350-400 g were used in all experiments. For GH potency assay the rats were anesthetized with sodium pentobarbital (60 mg/kg of body weight, administered i.p.), and 30 min. later the somatostatin analogs or saline were injected s.c. Blood samples were drawn from the jugular vein 15 min. after injection, and plasma was separated and assayed for GH by RIA according to MEYERS et al. (1980). The potencies were expressed as the percentage of somatostatin activity.

**Table 1**
**Structure and GH inhibitory activities *in vivo* of somatostatin analogs.**

| Code No. | Structure | GH inhibition* |
|---|---|---|
| SS-14 | Ala-Gly-c[Cys-Lys-Asn-Phe-PheTrp-Lys-Thr-Phe-Thr-Ser-Cys] | 1 |
| 117 | D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-NH-C$(CH_2OH)_2CH_3$ | 6 |
| 118 | D-Tyr-c[Cys-Phe-D-TrpLys-Thr-Cys]-NH-C$(CH_2OH)_2CH_3$ | 8.5 |
| 120 | D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-NH-CH$(CH_3)_2$ | 1.5 |
| 121 | D-Tyr-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-NH-CH$(CH_3)_2$ | 2.3 |
| 122 | D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-NH-$CH_2CH_2OH$ | 54 |
| 123 | D-Tyr-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-NH-$CH_2CH_2OH$ | 49 |
| 125 | D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-NH-$CH_2CH_2NH_2$ | 23 |
| 126 | D-Tyr-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-NH-$CH_2CH_2NH_2$ | 27 |

*Relative to somatostatin tetradecapeptide set at 1.0

**GH time-course assay**. Thiamylal (50 mg/kg, i.p.) and morphine sulphate (20 mg/kg, s.c.) were used as anesthetics and to maintain elevated GH levels (Cai et al. 1986). The animals were injected with thiamylal and then morphine was administered twice, 90 and 210 min after the injection of thiamylal. The analogs, in a dose of 1 μg/kg, and somatostatin, in a dose of 20 μg/kg (s.c.), were given 120 min after the induction of anesthesia. Blood was collected from the jugular vein 15, 30, 60, 120, 180, and 240 min. after the injection of the analog or somatostatin.

## Results

Heptapeptide analogs of octreotide with various substituted amides at the C-terminal and D-Phe or D-Trp at the N-terminal were synthesized by solid-phase method, purified and tested *in vivo* to evaluate the effect of these substitutions on the biological activity.

The crude products were obtained in about 70 % yields, on the basis of analytical HPLC. Disulfhydryl peptides were oxidized with iodine and purified by gel filtration on Sephadex G-10, followed by chromatography on Sephadex LH-20. The purity of the peptides was checked by analytical HPLC. In all cases the purity was found to be about 95 %, based on UV absorbance at 214 nm. The purified heptapeptides were also characterized by amino acid analyses, which gave the expected composition and by FAB-MS.

The native somatostatin possesses a wide range of biological activities. In our structure-activity relationship studies we concentrated on the inhibition of the release of growth hormone. The inhibitory effect of all new heptapeptide analogs on GH release *in vivo* in sodium pentabarbitol-anesthetized rats was measured. All new analogs showed greater potency in inhibiting GH secretion *in vivo* than somatostatin, with analogs 122 and 123 being the most potent analogs in this series (54 and 49 times more potent than somatostatin, respectively). The results of the inhibition of growth hormone release for all new analogs are shown in Table 1.

The time-course study, performed for all new peptides demonstrated that **122** was not only the most active analog, but also the longest-acting one. The GH inhibitory effect was observed for a period of 3 hours, at a dose of 1 μg/kg of body weight in contrast to a short duration of action of somatostatin given in a dose of 20 μg/kg of body weight (Fig. 1).

## Discussion

The synthetic heptapeptides reported here are based on octreotide and contain functional fragment Phe-D-Trp-Lys-Thr (corresponding to residues 7-10 of somatostatin), which was found to be an essential pharmacophore of somatostatin and its analogs (Veber et al. 1981). The conformational constraint endowed by the disulfide bridge allows the main functional fragment of the analogs to attain a bioactive conformation. However, it was found that hexapeptide structures consisting only of these two elements show low biological activity. The compound c[Cys-Phe-D-Trp-Lys-Thr-Cys]-$NH_2$ exhibited only 1.4% of somatostatin activity *in vivo* (Vale et al., 1979). Incorporation of D-Phe at the N-terminus and Thr-

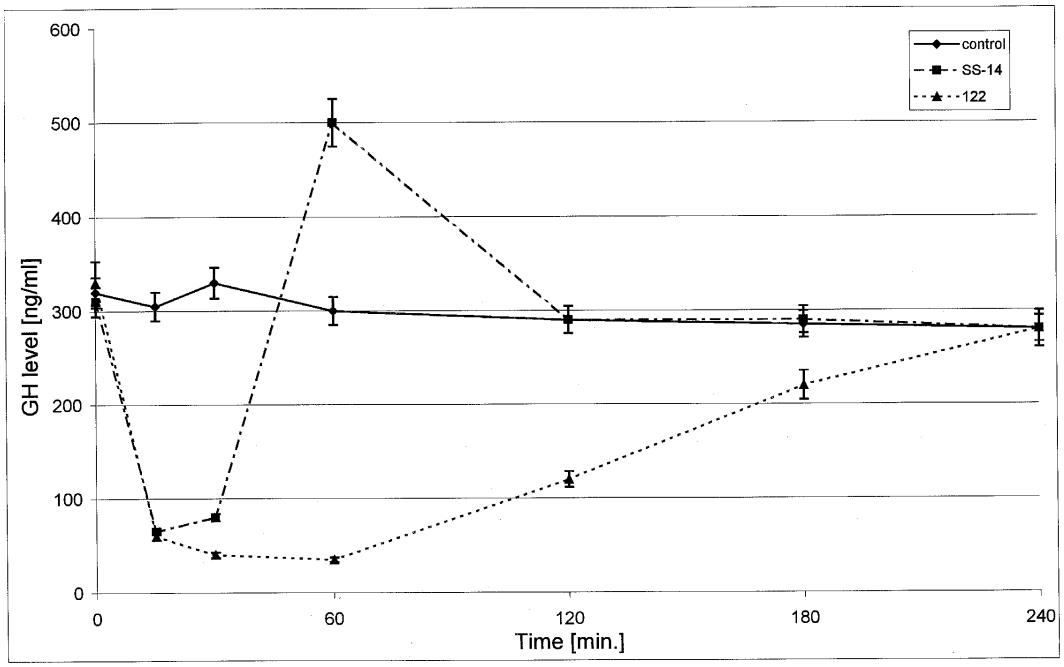


**Fig.1.**
**Time-course of inhibition of thiamylal/morphine-stimulated GH secretion in rats by somatostatin (SS-14) and heptapeptide analog** 122, **D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-NH-$CH_2CH_2OH$. Analog 122 was injected s.c. at a dose of 1 μg/kg of body weight and somatostatin was injected s.c. at 20 μg/kg of body weight at time 0. Control rats were injected with isotonic saline. Values shown are means ± SEM (n=5).**

ol or Thr-$NH_2$ at the C-terminus greatly increased the GH release inhibitory effect (Bauer et al., 1982).

Based on these results we decided to replace exocyclic residue (Thr-ol or Thr-$NH_2$) by a series of substituted amides on the C-terminal Cys residue. D-Phe or D-Tyr were used alternatively at the N-terminus. The resulted heptapeptide amides showed a GH-release inhibitory potency up to 54 times greater than that of somatostatin. The most potent analog of this series was found to be compound 122, D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-NH-$CH_2CH_2$-OH (Table 1), with the activity 54 times greater than somatostatin. Peptide 125, D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-NH-$CH_2CH_2$-$NH_2$ was 23 times more potent than somatostatin. Surprisingly, the analog 117, D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-NH-C($CH_2$-OH)$_2CH_3$ with two hydroxy groups, like in octreotide, was only 6 times more potent than somatostatin. The least potent analog 120, D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-NH- $CH(CH_3)_2$ had a hydrophobic substituent at the C-terminus and was almost equipotent with somatostatin. It seems that hydrophilic C-terminus is more advantageous for the potency of somatostatin analogs.

The pairs of peptides containing D-Phe or D-Tyr at the N-terminus all showed similar potencies. It appears that the N-terminal residue of the analogs is not directly involved in the receptor recognition, but more likely participates in the internal stabilization of the peptides through its aromatic side groups.

Analog 122, the most potent one of this series showed not only a high potency but also a long duration of action for inhibition of GH release (Fig. 1).

For a full estimation of biological activity of the new somatostatin analogs the binding experiments should be performed in the future to find their binding affinities at five somatostatin receptor subtypes.

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