SYNTHESIS OF A NONREDUCIBLE CYCLIC ANALOG OF SOMATOSTATIN HAVING ONLY GROWTH HORMONE RELEASE INHIBITING ACTIVITY

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SUMMARY: A nonreducible cyclic analog of somatostatin (SRIF) was prepared by a combination of solid phase and solution peptide synthesis. The compound, γ-Abu-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Asp-OH, was tested for its effect on the release of growth hormone, glucagon and insulin in rats. It significantly suppressed pentobarbital-stimulated growth hormone release but showed no effect on arginine-stimulated glucagon or insulin release. The linear form, NH_2-γ-Abu-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Asp-OH, was also prepared and tested *in vivo*. It was shown to have only slight activity.

Somatostatin is a hypothalamic peptide having the following sequence:

H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH (1).

SRIF exhibits a multitude of activities, among them; inhibition of release of growth hormone, glucagon and insulin (2). Our attention was focused on these activities. A recent publication (3) has reported that the disulfide bond in somatostatin may be replaced by an ethylene bridge without qualitatively changing the biological activity. An earlier report from our laboratories (4) indicated that somatostatin could be modified to lower only growth hormone and insulin levels without significantly affecting plasma glucagon. We report now the synthesis of a nonreducible cyclic analog (VI, see Figure 1) having remarkable specificity for the suppression of rat growth hormone release at dosages of 20-5,000 μg/kg (5) and compare its biological activity with that of the linear, non-sulfhydryl containing analog (VII).

Strategy for the assembly of the cyclic and linear peptide (VI and VII) is outlined in Figure 1. The resin bound peptide (I) was prepared by standard solid phase methods (6,7). The protected peptide was then cleaved from the resin by hydrazinolysis (8) to yield the protected hydrazide (II). Conversion of the hydrazide, II, to the azide and reaction *in situ* (9) with

Abbreviations used: γ-Abu, γ-aminobutyric acid; Boc, t-butyloxycarbonyl; Bzl, benzyl; 2-ClZ, 2-chlorobenzyloxycarbonyl; OBu^t, t-butyl ester; OBzl, benzyl ester; DCC, dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; DMF, dimethylformamide; TFA, trifluoroacetic acid.

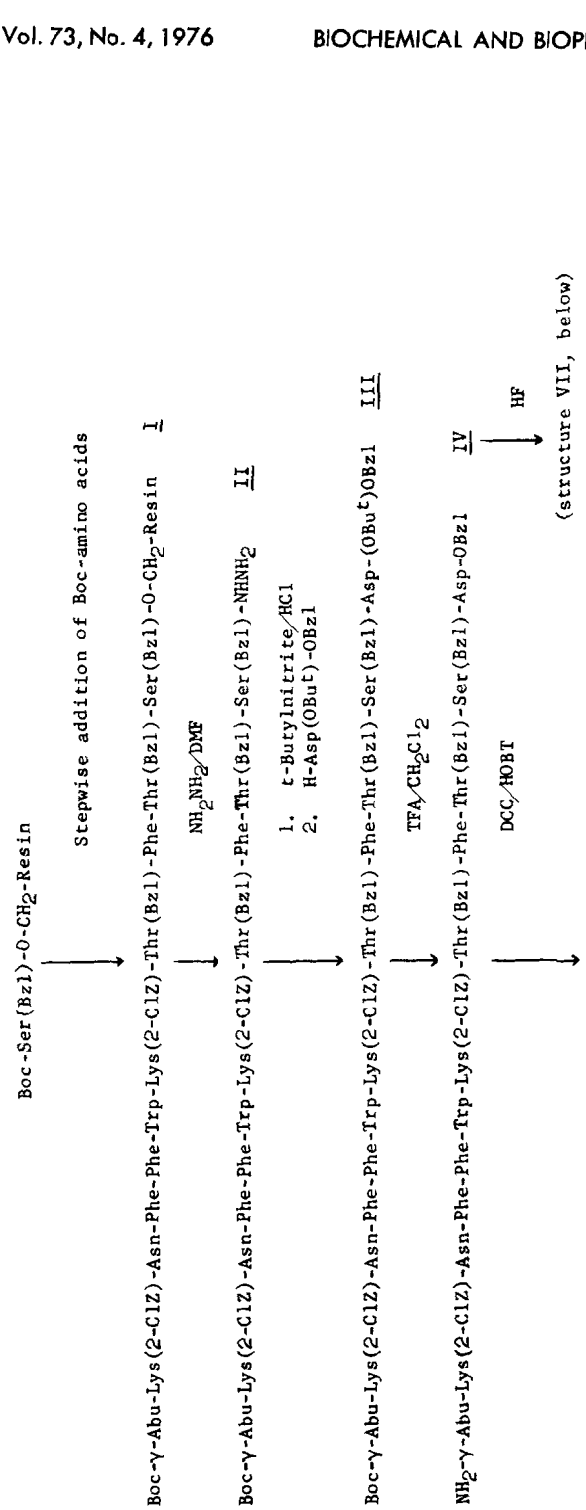

Figure 1. Outline of Synthesis of Cyclic and Linear Analogs (VI, VII)

Asp(OBu^t)-OBzl gave the dodecapeptide III. Trifluoroacetic acid treatment of III yielded the partially deprotected IV. In order to optimize the yield of desired product a high dilution cyclization was carried out in the presence of dicyclohexylcarbodiimide and hydroxybenzotriazole to give the cyclic protected peptide (V). Removal of all side chain protecting groups on intermediates IV and V was accomplished by the use of liquid hydrogen fluoride (10). The products, VI and VII, were purified by gel filtration and partition chromatography. The elution volumes of these products on Sephadex were consistant with monomeric structures. Purity of the peptides was established by amino acid analysis and homogeneity in three TLC systems.

METHODS AND MATERIALS

Synthetic Procedure (I). Boc(Bzl) serine (4.0 g, 1.5 m moles of amino acid) prepared from chloromethylated polystyrene (1% cross linking) by the method of Monahan (11) was placed in the reaction vessel of a Beckman 990 peptide synthesizer. The following schedule for deprotection, neutralization and coupling for the introduction of each amino acid was employed: (a) three washes with CH_2Cl_2; (b) removal of Boc protection with 30% TFA-CH_2Cl_2 (0.5% dithioerythritol) for 5 and 25 minute treatments; (c) six washes with CH_2Cl_2; (d) neutralization by three treatments with 15% triethylamine in DMF; (e) six washes with CH_2Cl_2; (f) addition of 6.0 m moles of protected amino acid in 50% DMF-CH_2Cl_2; (g) addition of 6.6 m moles of diisopropylcarbodiimide in two equal portions over a 30 minute period followed by mixing for four hours. All washes were 1.5 minutes unless noted otherwise. The following amino acids were introduced consecutively: Boc-Thr(Bzl), Boc-Phe, Boc-Thr-(Bzl), Boc-Lys(2-ClZ), Boc-Trp, Boc-Phe, Boc-Phe, Boc-Asn-ONp, Boc-Lys(2-ClZ), Boc-γ-Abu. The asparagine residue was incorporated via its p-nitrophenyl-ester in the presence of a catalytic amount of acetic acid and the coupling allowed to proceed for 24 hours. At the completion of the synthesis the resin was washed with methanol and dried *in vacuo* (7.1 g).

Hydrazinolysis (II). The protected peptide resin (7.1 g) was suspended in DMF (70 ml) and stirred with 95% hydrazine (3.0 ml) for 18 hours at 25°, filtered and washed with DMF. The filtrate was concentrated under reduced pressure and the resulting oil treated with water to yield a white precipitate (2.4 g).

Dodecapeptides (III and IV). A solution of the above described protected undecapeptide hydrazide, (2.1 g) in a mixture of dimethylsulfoxide (4.0 ml)-DMF (50 ml) was cooled (-30°C) and 1.0 N HCl in tetrahydrofuran (2.70 ml) added followed by the addition of t-butylnitrite (0.16 ml). After stirring for 30 minutes (-15°) a solution of H-Asp (O-Bu^t)-OBzl (0.36 g) in DMF (3 ml) was added and the pH adjusted to 7.5 with triethylamine. The reaction was stirred at 0° for 18 hours, concentrated under reduced pressure and the remaining oil triturated with citric acid (0.5 M). The precipitate was filtered, washed with water and dried (2.28 g). The protected dodecapeptide (2.28 g) was treated with a 30% solution of TFA-CH_2Cl_2 (0.5% dithioerythritol) for 40 minutes (25°). Following the addition of anhydrous ether a white precipitate was filtered and dried (2.2 g).

Protected Cyclic Peptide (V). To a cooled (0°) solution of the above described peptide, IV, (1.1 g in 300 ml of 2:1 DMF-CH_2Cl_2) was added 1-hydroxybenzotriazole monohydrate (0.73 g) followed by the addition of DCC (0.78 g). The reaction (adjusted to pH 7.5 with triethylamine) was stirred

at 0° for 3 hours then at 25° for 72 hours. Following filtration the reaction was concentrated under reduced pressure and the remaining oil triturated with a saturated solution of $NaHCO_3$. The white precipitate was filtered, washed thoroughly with water, 10% citric acid and water, yield, 2.1 g.

Deprotection (VI). The protected peptide, IV, (1.0 g), anisole (5 ml) and HF (50 ml) were stirred at 0° for 45 minutes. Excess HF was removed *in vacuo*. The product was dissolved in acetic acid (1 N) extracted with ether and the aqueous portion lyophilized (0.85 g).

Purification (VI). The lyophilized product (0.85 g) was purified on a column (2.5 x 200 cm) of Sephadex G-25 (fine) in 2N acetic acid. Fractions 180-190 (4.2 ml each) were lyophilized (0.20 g) and applied in upper phase n-butanol-acetic acid-water (4:1:5) on a column (2.5 x 100 cm) of Sephadex G-25 (medium) which had been equilibrated with lower phase, followed by upper phase. Fractions 95-120 were combined to yield 125 mg of homogenous product. R_f (cellulose), in (upper phase) n-butanol-water-acetic acid (4:1:5), 0.64; R_f, isoamyl alcohol-pyridine-water (7:7:6), 0.65; R_f, n-butanol-pyridine-acetic acid-water (4:1:1:2) 0.70. Amino acid analysis of a methanesulfonic acid hydrolysate gave Asp 1.90; Thr 2.06; Ser 0.85; Phe 3.00; Lys 2.10; Trp 0.85; γ-Abu 1.03.

Linear Peptide (VII). Deprotection and purification of the linear product was as described for the above cyclic material (V). R_f (cellulose) 4:1:5, 0.56; R_f 7:7:6, 0.56; R_f 4:1:1:2, 0.58. Amino acid analysis: Asp 1.85; Thr 2.03; Ser 0.90; Phe 3.00; Lys 2.05; Trp 0.88, γ-Abu 0.95.

Bioassay. *In vivo* hormone secretion was measured in groups of 8-10 male Charles River CD rats weighing about 250 g after stimulation of GH secretion by intraperitoneal injection of Na pentobarbital (50 mg/kg; zero time) and of glucagon and insulin secretions by intracardiac arginine (150 mg/rat; + 25 minutes). Somatostatin or its analog in saline, or saline alone, was injected subcutaneously at + 15 minutes into experimental rats or matched controls. The rats were decapitated at + 30 minutes, and trunk plasma, collected in Trasylol-EDTA, (12) was assayed for the three hormones by double antibody radioimmunoassays, using GH reagents kindly provided by the NIAMDD, commercial insulin reagents, and Unger 30K antiglucagon serum.

RESULTS AND DISCUSSION

Both analogs (VI and VII) were tested for their effect on growth hormone, insulin and glucagon levels (Table 1). The cyclic form (VI) demonstrated GH inhibitory activity without significantly affecting insulin or glucagon at a dosage range of 20-5,000 μg/kg. The linear form (VII) was inactive at all but the maximum dose level tested (5,000 μg/kg).

In three experiments with (VI) on glucagon secretion a small (20-29%) but statistically insignificant lowering can be seen. With insulin secretion, again a small lowering can be observed (11-29%), only one of which is statistically significant — at the maximum dosage (5,000 μg/kg). In experiment 5 there is a significant insulin lowering (71%) at a dosage of 4,000 μg/kg.

TABLE 1

Inhibition of Release of Growth Hormone, Glucagon, and Insulin by Somatostatin (SRIF) and Analogs

Exp.	Peptide	Dose μg/kg	Plasma Hormone Levels (M ± SEM) GH ng/ml	Glucagon pg/ml	Insulin μU/ml
1	Control	-	261 ± 43	21 ± 5	184 ± 14
	SRIF	200	73 ± 13**	5.7 ± 2**	112 ± 11**
	VI	4000	95 ± 17**	16 ± 4	164 ± 9
2[a]	Control	-	271 ± 25		
	SRIF	20	94 ± 32**		
	VI	100	109 ± 33**		
	VI	20	184 ± 29**		
3[a]	Control	-	350 ± 46		
	SRIF	10	193 ± 29**		
	VI	100	158 ± 23**		
4[a]	Control	-	227 ± 32		
	VI	100	44 ± 8**		
	VII	200	223 ± 30		
	VII	1000	155 ± 33		
5	Control	-	129 ± 20	62 ± 8	252 ± 16
	VI	4000	31 ± 7**	46 ± 6	179 ± 14**
	VII	4000	111 ± 19	66 ± 5	236 ± 18
6	Control	-	184 ± 23	55 ± 6	245 ± 24
	VI	5000	23 ± 1**	44 ± 10	210 ± 17
	VII	5000	63 ± 14**	63 ± 6	226 ± 15

** $p < 0.01$
* $p < 0.05$ compared to saline controls by analysis of variance.
[a] arginine injection omitted.

We tend to disregard this result based on repeated experiments at 4,000 and 5,000 μg/kg.

The demonstrated lack of activity for the linear analog (VII) suggests that the cyclic form of SRIF may also be a necessary structural requirement for significant biological activity. The reported activity of dihydrosomatostatin (13) could be a result of intramolecular oxidation to somatostatin under conditions of the bioassays (14).

Somatostatin analogs with dissociation of biological activities may prove to be of therapeutic value in the treatment of diabetes and acromegaly.

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