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Superactive octapeptide somatostatin analogs containing tryptophan at position 1

(solid-phase synthesis/structure–activity relationship/somatotropin)

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ABSTRACT **We synthesized a series of octapeptide analogs of somatostatin, containing N-terminal tryptophan or another amino acid followed by the hexapeptide sequences Cys-Phe-D-Trp-Lys-Thr-Cys or Cys-Tyr-D-Trp-Lys-Val-Cys and a C-terminal threoninamide or tryptophanamide. After purification by HPLC, the inhibitory activities of these analogs on the release of growth hormone (somatotropin) in rats were determined *in vivo*. The eight octapeptides with an N-terminal tryptophan residue were found to have a greater inhibitory effect than somatostatin. The most potent of these analogs, D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2, was 94.3 times more active than somatostatin. The other analogs, in order of decreasing potency, were Ac-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2, D-Trp(For)-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2, D-Trp-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2, Ac-Trp(For)-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2, Ac-Trp-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2, D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Trp-NH_2, and D-Trp-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2. The growth hormone inhibitory activity of these analogs was from 53.7 to 11.6 times greater than that of somatostatin. The octapeptides containing D- or L-tryptophan at the N-terminus, phenylalanine at position 3, and threonine at position 6 exhibited a greater inhibitory effect on growth hormone release than that of the analogs with tyrosine and valine at positions 3 and 6, respectively. Substitution of D-tryptophan for D-phenylalanine at the N-terminus in the octapeptide containing phenylalanine in the third, threonine in the sixth, and threoninamide in the C-terminal position also increased the growth hormone-release inhibitory activity. Time-course assay showed that D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2 (RC-98-I), in a dose of 1 μg/kg of body weight, inhibited the release of growth hormone for at least 3 hr. In view of their high activity and prolonged duration of action, some of these analogs could be useful clinically.**

Tetradecapeptide somatostatin is of little therapeutic value, because of its short half-life and broad spectrum of biological actions (1). Earlier structure–activity studies on somatostatin revealed that the entire molecule was not required for the maintenance of the biological activity of this peptide (2). Some somatostatin analogs containing modification of Trp-8 showed higher potency, but they exhibited a rapid inactivation *in vivo* (3, 4). From a number of cyclic analogs synthesized by Veber *et al.* (5, 6), the hexapeptide cyclo(-*N*-Me-Ala-Tyr-D-Trp-Lys-Val-Phe-) was found to be 50–100 times more potent than somatostatin in tests on the inhibition of growth hormone (GH, somatotropin), insulin, and glucagon release *in vitro*.

Another potent somatostatin analog is the octapeptide D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol) (7). This analog has not only a greater potency and duration of action on GH release but also a more selective effect on insulin and glucagon release. The suppressive action of this compound on GH release was found to be more selective than the inhibitory effect on insulin and glucagon secretion (7). Various analogs related to this octapeptide but containing tyrosine and valine at positions 3 and 6, respectively, and threoninamide or tryptophanamide at position 8 were synthesized in our laboratory (8, 9). These analogs showed a high potency and protracted duration of action on the inhibition of GH release *in vivo*. This paper reports the synthesis and the evaluations of biological activity of octapeptide somatostatin analogs containing various N-terminal substitutions.

MATERIALS AND METHODS

Synthesis. The analogs were synthesized in a Beckman model 990 automatic peptide synthesizer or in the usual glass reaction vessels, using standard solid-phase procedures (10). Benzhydrylamine resin (0.50 mmol/g, Bachem, Torrance, CA) was used as starting material. Amino acids (Bachem) were coupled as their N^{α}-*tert*-butoxycarbonyl (Boc) derivatives. The reactive side chains were protected as follows: serine and threonine, with benzyl; cysteine, with 4-methylbenzyl; lysine, with 2-chlorobenzyloxycarbonyl; tyrosine, with 2-bromobenzyloxycarbonyl. Stepwise coupling using carbodimide was performed in methylene chloride or other solvents. After the incorporation of tryptophan, 2-mercaptoethanol was added to the 50% CF_3COOH/50% CH_2Cl_2 (vol/vol) solution in all subsequent deblocking steps. The octapeptide amides were cleaved from the resin with simultaneous deprotection using liquid HF with anisole. The disulfhydryl peptides were cyclized in 95% CH_3COOH by using iodine (11).

Purification. The disulfhydryl or disulfide peptides were subjected to gel filtration on a 700 × 25-mm Sephadex G-15 (fine) column, in 30% CH_3COOH. The main fraction was further separated in a reversed-phase HPLC system, using a Dynamax macro C_{18} (12-μm particle size) 250 × 21-mm column (Rainin Instruments, Woburn, MA) and gradient elution (solvent A, 0.1% aqueous CF_3COOH; solvent B, 0.1% CF_3COOH in 70% CH_3CN).

Purity Control. The purity of the isolated substances was checked in a reversed-phase HPLC system, using a W-Porex $5C_{18}$ (5 μm) 250 × 4.6-mm column (Phenomenex, Rancho

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Abbreviations: Amino acid abbreviations are those of IUPAC, and all configurations are L except where noted otherwise; Boc, N^{α}-*tert*-butoxycarbonyl; For, formyl; GH, growth hormone.

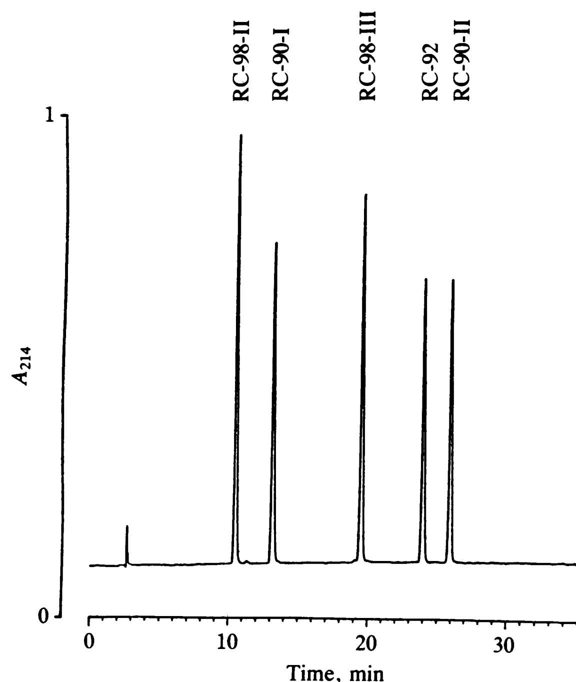

FIG. 1. HPLC of a mixture of D-Trp-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2 (RC-98-II), Ac-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2 (RC-92), Ac-Trp-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2 (RC-98-III), D-Trp(For)-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2 (RC-90-I), and Ac-Trp(For)-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2 (RC-90-II; For, formyl) applied to a W-Porex $5C_{18}$ (5 μm) Phenomenex column (250 × 4.6 mm) and eluted under gradient conditions as given below. Solvent A was 0.1% aqueous CF_3COOH; solvent B was 0.1% CF_3COOH in 70% aqueous CH_3CN. The elution gradient conditions were from 20% to 50% solvent B over 30 min; flow rate was 1.2 ml/min. UV detection was at 214 nm at room temperature.

Palos Verdes, CA), gradient elution, and the solvent system described above.

All chromatographic preparations were performed at room temperature (20°C), on a Beckman HPLC system, using a model 450 data system controller, two 114M solvent delivery modules, a 340 organizer, a 165 variable wavelength detector, and a Kipp and Zonen BD41 recorder. The detection wavelength was 214 nm.

Amino Acid Analysis. Amino acid analyses were performed in a Beckman 6300 amino acid analyzer, on samples that were hydrolyzed at 110°C for 20 hr in sealed, evacuated tubes with 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole.

GH Potency Assay. Adult male Sprague–Dawley rats weighing 200–350 g were used in all bioassays. 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 (12). The potencies were calculated by four-point assay (13) and expressed as the percentage of somatostatin activity.

GH Time-Course Assay. Chlorpromazine (10 mg/kg, s.c.; Thorazine, Smith Kline & French), sodium phenobarbital (60 mg/kg, i.p.; Baker, Phillipsburg, NJ), and morphine sulfate (20 mg/kg, s.c.; Eli Lilly) were used as anesthetics and to maintain elevated GH levels. The animals were injected with chlorpromazine and then, 15 min later, with phenobarbital. Morphine was administered twice, 90 and 210 min after the injection of chlorpromazine. The analog D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2 (RC-98-I), in a dose of 1 μg/kg, and somatostatin, in a dose of 20 μg/kg, were given 120 min after the initiation 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

Various octapeptide amides related to D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol) (7) but with different N-terminal and C-terminal substitutions were synthesized by automated solid-phase methods, purified, and tested *in vivo*. The crude products were obtained in about 65% yields, on the basis of analysis by HPLC. After gel filtration and preparative purification by HPLC of the crude octapeptides, single and symmetrical elution peaks were obtained, some of which are shown in Fig. 1. The purity of the octapeptides was found to be at least 95%, based on their UV absorbance at 214 nm. The spectral analysis of the octapeptides with a formyl group on the indole of Trp-1 showed an absorption maximum at 240 nm. Those peptides lacking a formyl group at the N-terminus (Figs. 2 and 3) showed minimum absorption at 240 nm. Our studies indicate that the formyl group at the N-terminus is not split in 12 hr at pH 7.4 in 0.15 M NaCl solution.

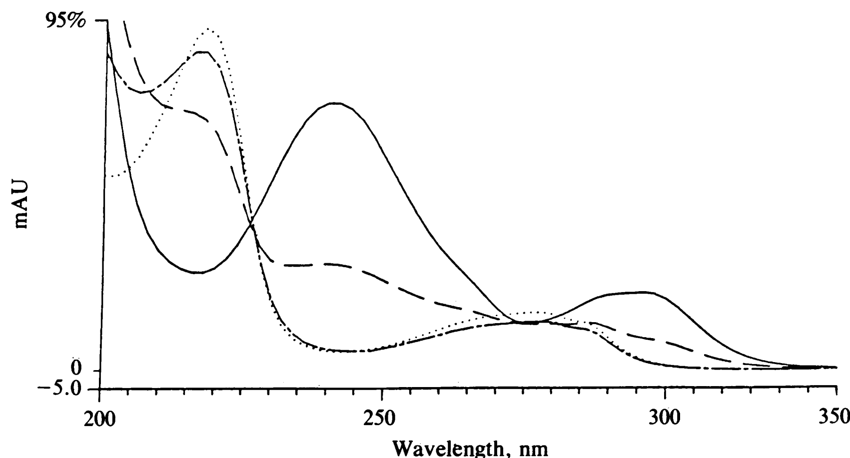

FIG. 2. Spectral analysis of Boc-Trp (· · · · · ·), Boc-Trp(For) (———), Ac-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2 (RC-92) (—·—·—), and Ac-Trp(For)-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2 (RC-90-II) (— — —) between 200 and 350 nm. Boc-Trp(For) and RC-90-II show an absorption maximum at 240 nm, whereas Boc-Trp and RC-92 (without formyl groups) show an absorption minimum at 240 nm. mAU, absorbance milliunits in %.

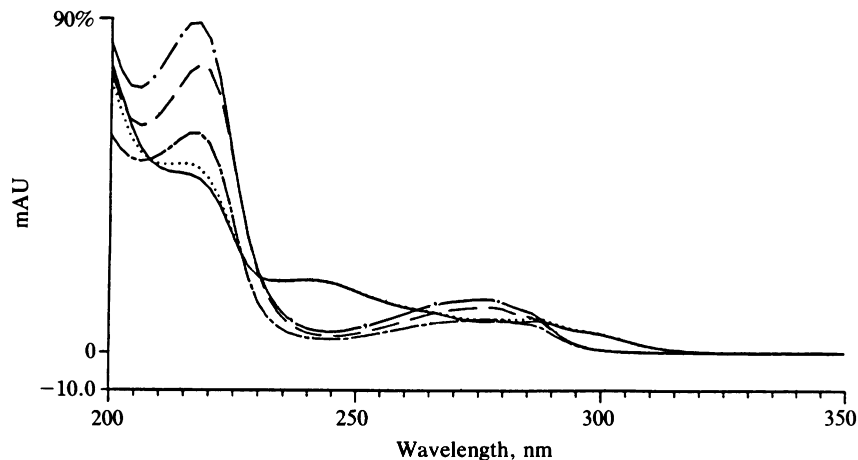

FIG. 3. Spectral analysis of D-Trp-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2 (RC-98-II) (—·—·—), Ac-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr--NH_2 (RC-92) (— — —), Ac-Trp-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2 (RC-98-III) (– – – – –), D-Trp(For)-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr--NH_2 (RC-90-I) (·······), and Ac-Trp(For)-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2 (RC-90-II) (———) between 200 and 350 nm. The peptides (RC-90-I and RC-90-II) containing a formyl group were found to have an absorption maximum at 240 nm, whereas the octapeptides without a formyl group were found to have minimum absorption at 240 nm. mAU, absorbance milliunits in %.

The inhibitory effect of D-Trp-Cys-Phe-D-Trp-Lys-Thr--Cys-Thr-NH_2 (RC-98-I-2H) on GH release was found to be 92.6 times greater than that of somatostatin, in our *in vivo* assay (Table 1). This analog was found to be the most potent in the series of peptides synthesized, but other analogs listed in Table 1 exhibited a potency at least 5 times greater than that of somatostatin.

The series of octapeptides in disulfide form, containing D-Trp, Ac-Trp, D-Trp(For), or Ac-Trp(For) at the N-terminus, followed by the sequence Cys-Phe-D-Trp-Lys-Thr-Cys or Cys-Tyr-D-Trp-Lys-Val-Cys and a C-terminal Thr-NH_2 or Trp-NH_2 (Table 2) were synthesized in order to evaluate the contribution of N-terminal tryptophan to the biological activity (Table 3). The crude disulfhydryl octapeptides were oxidized with iodine to the disulfide form and were then subjected to gel filtration and HPLC purification. The peptides were eluted as single, symmetrical peaks. In all cases the purity was found to be at least 95%, based on UV absorbance at 214 nm (Table 2). Amino acid analysis of the acid hydrolysates of these peptides gave the expected composition (Table 2). The GH-release inhibitory effect of RC-98-I was found to be 94.3 times higher than that of somatostatin. This activity was similar to that of RC-98-I-2H, a disulfhydryl form of RC-98-I.

Ac-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2 (RC-92) was found to be 53.7 times more potent than somatostatin. D-Trp-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2 (RC-98-II) and Ac-Trp-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2 (RC-98-III), in which Phe-3 and Thr-6 were replaced by Tyr-3 and Val-6, had only about 50% of the GH-release inhibitory potency of the corresponding analogs, RC-98-I and RC-92, respectively. Insertion of a formyl group onto the indole group of the N-terminal D-Trp and Ac-Trp, as in the case of D-Trp(For)--Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2 (RC-90-I) and Ac--Trp(For)-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2 (RC-90-II), was found to produce GH-release inhibitory activity 52.4 and 49.5 times higher than that of somatostatin. RC-99-I and RC-99-II, containing D-Trp and Trp-NH_2 at positions 1 and 8, respectively, were less potent than RC-98-I and RC-98-II.

The time-course study demonstrated that RC-98-I was not only a superactive analog, but also a long-acting one. The GH inhibitory effect was observed for a period of at least 3 hr 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 (Fig. 4). An attempt was made to calculate the potency of the analogs by using integrated GH levels as a parameter of GH inhibitory activity. Mean serum GH levels were plotted arithmetically against time, and the area under the curve, compared to that of the saline-injected group, was calculated. The area indicating the total depression of the integrated serum GH level for RC-98-I was 84 times greater than that of somatostatin, after allowing for the difference in doses. This value is in good agreement with that obtained from four-point assays.

DISCUSSION

The synthetic octapeptides reported here, based in part on the somatostatin analog models of Veber *et al.* (14, 15), contain the functional fragments Phe-D-Trp-Lys-Thr or Tyr-

Table 1. Structure and GH inhibitory activities *in vivo* of somatostatin analogs (in disulfhydryl form)

Code no.	Structure	Inhibition*
RC-155-2H	Ac-Gly-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2	7.8
RC-131-2H	D-Ala-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2	17.5
RC-130-II-2H	D-Ser-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2	5.2
RC-130-III-2H	Ac-Ser-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2	6.4
RC-127-2H	D-Val-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2	7.9
RC-127-II-2H	D-Val-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2	5.7
RC-129-2H	D-Thr-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2	24.5
RC-98-I-2H	D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2	92.6
RC-98-II-2H	D-Trp-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2	34.6

*Relative to somatostatin tetradecapeptide, set at 1.0.

Table 2. Characterization and yields of somatostatin analogs

Code no.	Structure	Amino acid analysis, no. of residues								Purity,* %	Yield,† %
		Cys	Phe	Tyr	Trp	Lys	Thr	Val	NH_3		
RC-98-I	D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2	2.01	0.98		1.81	1.00	2.01		1.14	95	25.9
RC-98-II	D-Trp-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2	1.98		1.00	2.00	1.03	1.00	0.98	1.11	96	25.5
RC-92	Ac-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2	1.98	1.03		2.08	1.00	1.96		1.02	97	30.2
RC-98-III	Ac-Trp-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2	1.83		1.00	1.86	0.96	0.99	0.97	0.87	97	14
RC-90-I	D-Trp(For)-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2	1.86	1.00		2.00	0.99	1.98		0.99	96	36.4
RC-90-II	Ac-Trp(For)-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2	1.85	1.00		1.88	0.99	1.95		0.84	95	26.6
RC-99-I	D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Trp-NH_2	1.97	1.00		2.97	1.04	1.07		1.14	95	26.6
Rc-99-II	D-Trp-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2	2.07		0.99	3.04	0.99		1.03	0.95	96	21.7

*Based on the integration results of analytical HPLC runs monitored at 214 nm.
†Overall yield based on the molar equivalent of starting benzhydrylamine resin.

-D-Trp-Lys-Val, corresponding to residues 7–10 of somatostatin. The conformational constraint endowed by the two half-cystine residues allows the main functional fragment of the octapeptide to attain a bioactive conformation. However, the hexapeptide structures consisting only of these two components show low biological activity. The compound Cys-Phe-D-Trp-Lys-Thr-Cys-NH_2 exhibited only 1.4% somatostatin activity *in vivo* and less than 0.1% activity *in vitro* (7). Incorporation of D-phenylalanine in the N-terminal position and threoninol or threoninamide at the C-terminus greatly increases the GH-release inhibitory effect (7). Our earlier studies on octapeptide somatostatin analogs indicated that the replacement of C-terminal threoninol by threoninamide, or by a hydrophobic tryptophanamide residue, using relatively easy synthetic procedures, led to molecules with high GH-release inhibitory activity (8).

Based on these results, we decided to substitute the N-terminus of the octapeptides with other amino acids in order to study the structure–activity relationship. This resulted in peptides with a GH-release inhibitory potency from 5 to 100 times greater than that of somatostatin. The most potent of this series (Table 1) was found to be D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2 (RC-98-I), with an activity 94.3 times greater than that of somatostatin. It appears that the substitution of D-tryptophan at the N-terminus, in place of D-phenylalanine, preserves the biological activity of the analog. Because of a similar *in vivo* activity of octapeptides containing D or L isomers of tryptophan or phenylalanine at the N-terminus, it appears that the N-terminal residue of the octapeptide is not directly involved in the receptor-recognition process but more likely participates in the internal stabilization of the octapeptide through its aromatic side group. Therefore, complementary hydrophobic groups can significantly increase the biological activity of the analogs.

Table 3. Relative potencies of somatostatin analogs on inhibition of GH release *in vivo*, based on four-point assays

Code no.	No. of experiments	Inhibition of GH release*
RC-98-I	3	94.3 (70.5–190.1)
RC-98-II	2	49.8 (44.0–131.0)
RC-92	2	53.74 (30.3– 78.0)
RC-98-III	2	24.45 (8.5– 33.0)
RC-90-I	1	52.4 (20.9–131.5)
RC-90-II	2	49.55 (31.4– 86.4)
RC-99-I	1	19.4 (4.5– 82.2)
RC-99-II	1	11.6 (4.8– 27.8)

*Relative potency as compared to somatostatin tetradecapeptide, set at 1.0. Where more than one experiment was done, values are averages. The 95% confidence limits are given in parentheses.

The substitution of *p*-chloro-D-phenylalanine (9) for D-phenylalanine leads to a decrease in the GH-release inhibitory effect of the somatostatin analog. This phenomenon may be explained by the modified electron distribution of the phenol ring at the N-terminal residue rather than by the conformational changes in the structure caused by the halogen.

The formyl group on tryptophan can be released by HF in 1,2-ethanedithiol (16), resulting in RC-98-I or RC-92 from RC-90-I or RC-90-II. Spectral analysis at 240 nm of the formyl-containing peptides revealed that the formyl group on tryptophan was stable at pH 7.4. This formyl group may be stable in biological systems under the conditions similar to those of our bioassays. Although some formyl-substituted octapeptide analogs were found to have an increased renin inhibitory potency *in vitro* (17), our results with RC-90-I and RC-90-II showed no significant differences in the biological potencies of these octapeptide analogs *in vivo*, as compared to RC-98-I and RC-92.

Whereas the three analogs D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2 (RC-98-I), D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2 (RC-121), and D-Phe-Cys-Tyr-D-Trp-Lys-

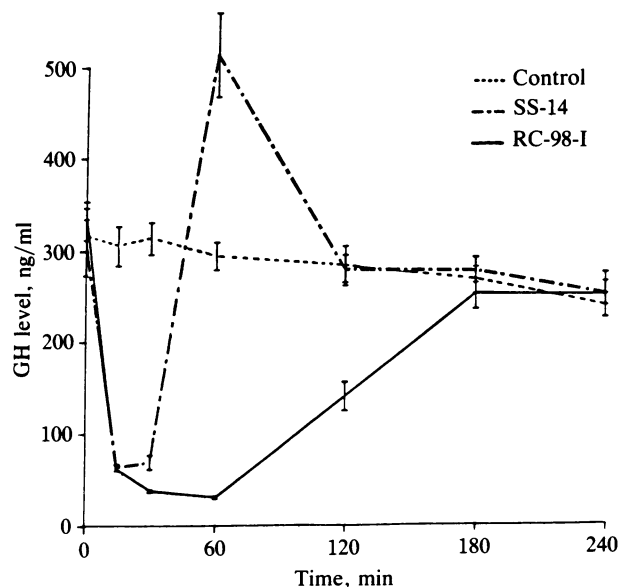

FIG. 4. Time course of inhibition of thiamylal/morphine-stimulated GH secretion in rats by somatostatin tetradecapeptide (SS-14) and its RC-98-I octapeptide analog. Analog RC-98-I was injected s.c. at a dose of 1 μg/kg of body weight and somatostatin was injected s.c. at 20 μg/kg of body weight at time zero. Control rats were injected with isotonic saline. Values shown are means ± SEM (n = 8).

-Val-Cys-Trp-NH_2 (RC-160) show a very high inhibitory activity on GH release, it was unexpected to find that a number of substituted compounds such as D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Trp-NH_2 (RC-99-I) and D-Trp-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2 (RC-99-II) had low activities *in vivo*.

Our previous results (8) showed that the synthetic octapeptide analogs containing Tyr-3, Val-6, and C-terminal threoninamide (RC-121) or Tyr-3, Val-6, and C-terminal tryptophanamide (RC-160) had 2–3 times greater potency in tests on inhibition of GH release than the related analogs with Phe-3, Thr-6, and C-terminal threoninamide (RC-102) or Phe-3, Thr-6, and C-terminal tryptophanamide (RC-95-I). The somatostatin octapeptide analogs containing D-tryptophan at the N-terminus, tyrosine and valine at positions 3 and 6 respectively, and threoninamide or tryptophanamide at position 8 show a significant decrease in biological activity *in vivo*. This demonstrates that the *in vivo* effect of a peptide is dependent not only on the amino acid content but also on the conformation of the molecule.

The differences between the *in vivo* and *in vitro* activities could be explained by the view that the *in vitro* potency of the somatostatin analogs might reflect, in part, the binding affinity between the hormone and the receptor. However, the *in vivo* situation is more complex. Thus, an octapeptide containing the fragment Tyr-D-Trp-Lys-Val shows about 30 times higher somatostatin activity *in vitro* than the related analog with the sequence Phe-D-Trp-Lys-Thr, but the biological effects of these two compounds are similar *in vivo* (18).

Various clinical trials carried out with D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol) (19) showed that it is effective in different endocrine-related disorders, including acromegaly. We previously reported (20) that octapeptides RC-121 and RC-160 have significant antitumor activities in animal models of prostate cancer and pancreatic cancer. Our *in vivo* studies with D-Trp-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2 show that this octapeptide analog, in addition to being superactive, also exerts prolonged inhibitory effect on GH release. High activity and long duration of action of some of the analogs reported here may make them useful clinically.

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