THE SYNTHESIS AND BIOLOGICAL ACTIVITY OF ALA^{3,14}-SOMATOSTATIN

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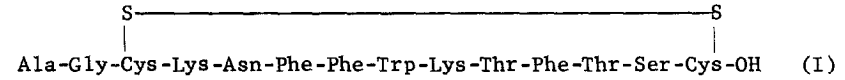
Summary: The tetradecapeptide H-Ala-Gly-Ala-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Ala-OH (Ala^{3,14}-somatostatin) an analog of the somatotropin release inhibiting factor (somatostatin SRIF) was synthesized by solid phase peptide methods. It shows somatotropin release inhibiting activity in vitro at 5 μg/ml concentration.

The Somatotropin Release Inhibiting Factor (SRIF, or somatostatin) from ovine hypothalamus was shown recently by Brazeau et al (1) to possess the sequence (I). The oxidation state of the two cysteines could not be decided unequivocally since the linear synthetic tetradecapeptide (II) was found as active as the native somatostatin in the inhibition of somatotropin release from monolayer cultures of dispersed pituitary cells. However, conversion to the disulfide form in the oxidizing environment of the incubating cell cultures or at the receptor site could account for the observed biological activity.

In our efforts to elucidate the structural requirements of the somatostatin (I) for the expression of its biological activity, we have synthesized the tetradecapeptide (III) (Ala^{3,14}-somatostatin) in which the cysteines at position 3 and 14 have been substituted by alanine. Obviously this analog cannot form a covalent cyclic structure, and any activity could be interpreted as evidence in favor of the linear sequence possessing inherent biological activity.



Abbreviations: BOC, tert-butyloxycarbonyl, 2-Cl-Cbz, 2-chlorobenzyloxycarbonyl, Bzl, benzyl, DCC, dicyclohexycarbodiimide, DMF, dimethylformamide.

Ala-Gly-Cys(SH)-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys(SH)-OH (II)

Ala-Gly-Ala-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Ala-OH (III)

Synthetic procedure: The solid phase methodology for the synthesis of peptides (2) was employed. Chloromethylated polystyrene resin (1% cross-linking) was esterified with BOC-Ala-OH and triethylamine in refluxing ethanol for 18 hours to give a substitution of 0.35 m moles alanine/gram of resin. The resin was transferred to a Merrifield vessel and deprotected with 50% trifluoroacetic acid in methylene chloride.

The following amino acid residues were introduced consecutively: BOC-Ser(Bzl)-OH, BOC-Thr(Bzl)-OH, BOC-Phe-OH, BOC-Thr(Bzl)-OH, BOC-Lys(2-Cl-Cbz)-OH (3), BOC-Trp-OH. All the couplings were carried out in a mixture of methylene chloride and DMF (not less than 2:1) using two equivalents of the corresponding N-protected amino acid and DCC (in two portions over a period of one hour) and for at least four hours at room temperature. The completion of the coupling was followed by the ninhydrin test of Kaiser et al (4) and quantitative amino acid analyses. In the event of positive ninhydrin test the coupling was repeated as above. The deprotection for the next step was achieved by 50% trifluoroacetic acid in methylene chloride.

After the incorporation of the tryptophan residue the deprotections were carried out by using 50% trifluoroacetic acid in methylene dichloride containing 5% 1,2-ethanedithiol while the couplings were achieved as before. The following amino acids were reacted consecutively: BOC-Phe-OH, BOC-Phe-OH, BOC-Asn, BOC-Lys(2-Cl-Cbz)-OH, BOC-Ala-OH and the dipeptide BOC-Ala-Gly-OH. In the case of L-asparagine it was coupled as the p-nitrophenyl ester in the presence of a catalytic amount of glacial acetic acid for four days at room temperature.

Cleavage and deprotection: The protected peptide resin was washed thoroughly with boiling methanol, dried and mixed with anisole (at least five equivalents per protecting group) then treated with liquid hydrogen fluoride (at least five times the volume of anisole) for 30 minutes at 0°C and 15 minutes at room

Table 1

Exp.	Concentration of (III) µg/ml	Somatotropin ng/ml
A	-*	128 ± 3
	50	32 ± 5**
	5	52 ± 6**
B	-*	272 ± 11
	1	245 ± 30

*Endogenous release
**$p < 0.01$

temperature. The hydrogen fluoride and anisole were removed as quickly as possible under reduced pressure and the residue was taken up in 1% acetic acid, filtered and lyophilized.

The crude product was purified by chromatography through (a) Bio-Gel P-2 (elution with 0.2 N-acetic acid), (b) Sephadex LH 20 (elution with a mixture of ethanol-0.2 N acetic acid, 4:1) and (c) Sephadex LH 20 (elution with 0.2 N-acetic acid).

The tetradecapeptide (III) was obtained as a white fluffy solid, $[\alpha]_D^{26}$ -44.1 (C 1.04, 1% AcOH) and found homogeneous on thin layer chromatography in three systems. R_f (n-butanol-water-acetic acid-pyridine, 30:24:6:20) 0.40, R_f (n-butanol water-pyridine, 3:1.5:2)0.15, R_f (ethyl acetate - pyridine-acetic acid-water, 5:5:1:3) 0.51.

Amino acid analysis: Ala (3) 2.88, Gly (1) 1.04, Lys (2) 1.98, Asp (1) 0.98, Phe (3) 3, Trp (1) 0.74, Thr (2) 1.66, Ser (1) 0.71 (numbers in brackets are the theoretical values). The overall yield was 0.54%.

Biological activity: Monolayer cultures of enzymatically dispersed cells from whole pituitaries of Charles River CD male rats were prepared and used essentially according to Vale et al (5) and Grant et al (6). The growth medium was Eagle's minimal essential medium (MEM) containing 10% fetal calf serum plus

standard amounts of glutamine and antibiotics, and the test medium was Earle's Balanced Salt Solution (EBSS) plus antibiotics. Treatments were each accorded to at least two culture dishes (always three for controls). After the 3-hour incubation at 37° in an atmosphere of 95% air, 5% CO_2, the supernatant solutions from each dish were assayed in duplicate for pituitary hormones by double antibody radioimmunoassays. The somatotropin assay employed NIAMDD-Porcine GH-P 522A for iodination, anti-rat GH lot V-30-D 1, kindly supplied by Dr. R.E. Grindeland of the Ames Research Center, Moffett Field, California, and NIAMDD-rat GH-RP-1 as standard. Results are shown in Table 1.

$Ala^{3,14}$-Somatostatin shows activity down to about 5 μg/ml. Since somatostatin itself shows activity down to about 0.5 ng/ml (7) the analog has approximately 0.01% of somatostatin's potency. The small but significant activity of the analog indicates that the disulfide bond of somatostatin very likely is not required for any reaction with the receptor. A cyclic system confers on the peptide enough rigidity to increase the population of conformations which are recognized by the receptor and trigger the biological response. Rudinger et al (8), Jost et al (9) and Katsoyannis et al (10) have come to similar conclusions regarding oxytocin and insulin analogs.

Work is in progress on the elucidation of structure-activity requirements of somatostatin.

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