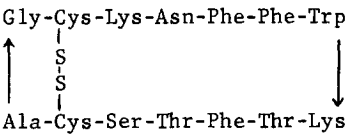
A BICYCLO-SOMATOSTATIN ANALOG, HIGHLY SPECIFIC FOR THE INHIBITION OF GROWTH HORMONE RELEASE

D. Sarantakis, J. Teichman, D.E. Clark, and E.L. Lien

Research Division, Wyeth Laboratories
Philadelphia, Pennsylvania 19101

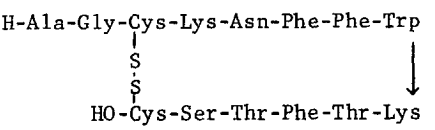
Received December 6, 1976

Summary: The tetradecapeptide somatostatin was cyclized by a combination of conventional and solid phase peptide synthesis methods, to a homodetic cyclic disulfide tetradecapeptide, Wy-40,391:



The analog inhibits the release of growth hormone (GH) in vivo without affecting either insulin or glucagon secretion. A correlation between binding affinity to the receptors and specificity is suggested.

Brazeau et al (1) recently reported the isolation and amino acid sequence of somatostatin, a tetradecapeptide disulfide which inhibits stimulated growth hormone secretion, as well as glucagon (2), insulin (3), gastrin (4), and secretin (5), secretion in mammals.



Somatostatin

Recently we showed (6) that des-Ala^1,Gly^2,Asn^5-somatostatin lowers plasma growth hormone and insulin without affecting plasma glucagon levels in vivo, this analog representing the first example of separation of activities in the somatostatin field. Subsequently we reported (7) the structure of two carbacyclic analogs of somatostatin which significantly suppressed pentobarbital-stimulated growth hormone release but showed no effect on arginine-stimulated glucagon or insulin release.

We describe here the synthesis of a bicyclic analog of somatostatin,

ISSN 0006-291X

Wy-40,391 which has good activity in inhibiting the release of growth hormone without affecting the insulin or glucagon secretion while retaining the amino acid sequence of the parent molecule.

Synthesis: The peptidoresin, Boc-Cys(SMBzl)-Ala-Gly-Cys(SMBzl)-Lys(ClCbz)-Asn-Phe-Phe-Trp-Lys(ClCbz)-Thr(Bzl)-Phe-Thr(Bzl)-Ser(Bzl)-O-Resin (II) was prepared from 1% cross-linked chloromethylated polystyrene which was esterified with Boc-Ser(Bzl)-OH by Gisin's cesium salt method (8). The polymeric ester was then treated according to schedule A for the incorporation of Boc-Thr(Bzl)-OH, Boc-Phe-OH, Boc-Thr(Bzl)-OH, Boc-Lys(ClCbz)-OH, Boc-Trp-OH, Boc-Phe-OH, and Boc-Phe-OH. In the case of the asparagine residue, the p-nitrophenyl ester, Boc-Asn-ONP, was employed in a ten-fold excess in DMF and in the presence of N-hydroxybenzotriazole as catalyst (9), after which Boc-Lys(ClCbz)-OH, Boc-Cys(SMBzl)-OH, Boc-Ala-Gly-OH, and finally Boc-Cys(SMBzl)-OH were added again according to schedule A.

Obviously we could initiate the synthesis from any convenient amino acid of the sequence of somatostatin; however, we took into consideration that the tryptophan and cysteine residues should be exposed to the minimum number of acidic treatments during the deprotection step, and in case of partial failure to cyclize in the subsequent step, the linear byproduct should not be somatostatin or a closely related analog. The peptidoresin (II) gave an acceptable amino acid analysis after hydrolysis with 12 N-aq. HCl-propionic acid (1:1).

Treatment of (II) with anhydrous hydrazine (50 equivs.) in DMF for 4 hours at 20°C afforded the tetradecapeptide hydrazide (III), mp=280-285°, $[\alpha]_D$ + 4.5 (1% DMF), Boc-Cys(SMBzl)-Ala-Gly-Cys(SMBzl)-Lys(ClCbz)-Asn-Phe-Phe-Trp-Lys(ClCbz)-Thr(Bzl)-Phe-Thr(Bzl)-Ser(Bzl)-$NHNH_2$ (III). This was treated with trifluoroacetic acid in the presence of anisole for 30 minutes in an ice-bath to remove the N-terminal, butyloxycarbonyl group, affording (IV) mp = 265-270° $[\alpha]_D$ + 0.5 (1% DMF) H-Cys(SMBzl)-Ala-Gly-Cys(SMBzl)-Lys(ClCbz)-Asn-Phe-Phe-Trp-Lys(ClCbz)-Thr(Bzl)-Phe-Thr(Bzl)-Ser(Bzl)-$NHNH_2$, 2 CF_3CO_2H (IV).

Table I

Properties of Bicyclo-Somatostatin Analogs

	Asp	Thr	Ser	Gly	Ala	Phe	Lys	Cys	Trp	$[\alpha]_D^{26}$	R_fA	R_fB
Wy-40,391	0.95	1.89	0.89	0.89	0.90	3	1.90	1.63	ND	-25°±1	0.56	0.74
Wy-41,524	0.98	1.89	1.02	1.06	0.99	3.12	2	1.54	ND	- 6°±2	0.44	0.70

TLC, System A, n-Butanol-water-gl. acetic acid, 4:1:1 v/v, System B, n-butanol-water-gl. acetic acid-pyridine, 30:24:6:20 v/v, Avicel precoated glass plates. Samples for amino acid analysis were hydrolyzed in 6 N-aqueous HCl, 109°C for 24 hours under nitrogen. Optical rotations - (C 1, 75% AcOH) and (C 2.4, 50% AcOH) correspondingly

This tetradecapeptide hydrazide was converted to the azide by Rudinger's method (10) and then cyclized under high dilution. The cyclization product consisted of the desired cyclic monomer and higher polymeric species and was not characterized but was globally deprotected with liquid HF in the presence of anisole followed by oxidation of the two sulfhydryl groups by air at pH 7.2. The crude material containing cyclo-somatostatin (I) was purified by gel filtration through Sephadex G-25 (2.5 x 200 cm) and eluted with 1 M-aq. AcOH. The cyclo-somatostatin disulfide emerged between 496 and 800 ml, (Table I). The mobility of the compound during the gel filtration makes it unlikely that we have a dimer. Dansylation also failed to show any free α-amino group.

An identical procedure was followed for the synthesis of Wy-41,524, which has the D-isomer in place of L-tryptophan.

Biological Testing: Male Charles River CD rats weighing 250-300 grams were injected with 50 mg/kg nembutal (i.p.). Fifteen minutes later they were injected subcutaneously with either Wy-40,391 in saline or saline alone, followed in ten minutes with administration of 0.5 ml of arginine (300 mg/ml, pH 7.2) via cardiac puncture. Trunk blood was collected five minutes after the arginine injection by rapid decapitation (12 mg of versene and 6000 units of Trasylol added per tube). Each sample was assayed in quadruplicate for growth hormone, glucagon and insulin by specific double antibody radio-

Table II

Effects of Wy-40,391 and Wy-41,524 on Suppression of Growth Hormone, Insulin, and Glucagon at 15 Minutes in Rats

Exp.		Dose µg/kg	Plasma Hormone Levels (M ± SEM) GH ng/ml	Insulin µU/ml	Glucagon pg/ml
A	Control	-	291 ± 47	272 ± 39†	33 ± 2
	Somatostatin	200	40 ± 4*	161 ± 21†	17 ± 2*
	Wy-40,391	3000	40 ± 4*	308 ± 30	29 ± 4
B	Control	-	505 ± 139	-	-
	Wy-40,391	1500	82 ± 19*	-	-
C	Control	-	334 ± 81	-	-
	Wy-40,391	200	58 ± 11*	-	-
D	Control	-	334 ± 81†	-	-
	Wy-40,391	20	141 ± 28†	-	-
E	Control	-	222 ± 27	346 ± 28	49 ± 5
	Wy-41,524	400	58 ± 6*	228 ± 10*	29 ± 4

* $p < 0.01$, † $p < 0.05$ compared to saline controls by analysis of variance. 10 Rats per group in experiments A and B, 8 rats per group in experiments C, D and E.

immunoassays. Growth hormone was assayed using NIAMDD reagents. Porcine insulin antisera was obtained from Chappell Laboratories and ^{125}I-labelled insulin from New England Nuclear. Glucagon was determined using Unger 30K antisera and ^{125}I-glucagon purchased from Nuclear Medical Laboratories.

Results and Discussion: The effect of Wy-40,391 (in doses from 20 µg/kg to 3,000 µg/kg) on plasma growth hormone levels is shown in Table II. Growth hormone was significantly reduced by levels of the peptide as low as 20 µg/kg. The minimal effective dose of somatostatin for lowering growth hormone in this assay is 2 µg/kg (11), or about one tenth the level of Wy-40,391. No suppression of glucagon or insulin was observed at the highest level of Wy-40,391 studied (3,000 µg/kg), while in the same experiment somatostatin (at 200 µg/kg) lowered both hormones significantly (Table II,

Schedule A

Procedure for the Deprotection and Coupling of Amino Acid Derivatives.

1. Wash with CH_2Cl_2 x 3
2. Treat with CF_3CO_2H-CH_2Cl_2-1,2-ethanedithiol (1:1:4%) for 5 minutes
3. Treat as in 2 for 25 minutes
4. Wash with CH_2Cl_2 x 3
5. Wash with DMF
6. Treat with 12% triethylamine in DMF twice for 3 minutes
7. Wash with DMF
8. Wash with CH_2Cl_2 x 3
9. Treat with 4 equivalents of the corresponding amino acid derivative in CH_2Cl_2-DMF and 4 equivalents of N-hydroxybenzotriazole and stir for 5 minutes.
10. Add in two portions 5 equivalents of N, N'-diisopropylcarbodiimide dissolved in CH_2Cl_2 and over a period of 30 minutes. Reaction time 10-12 hours.
11. Wash with DMF x 3
12. Wash with CH_2Cl_2 x 3

exp. A). These <u>in vivo</u> results demonstrate clearly that Wy-40,391 is more specific in suppressing growth hormone release than is somatostatin.

We have not examined the specificity of the compound towards other activities of somatostatin. The absence of a free α-amino group or carboxyl group suggests that these groups are not essential for the growth hormone inhibiting activity of somatostatin.

The cyclization of the peptide chain of somatostatin in our analog restricts to a certain extent the flexibility of the molecule. There is the possibility that the insulin and glucagon receptors are much more demanding with regard to conformational integrity and do not respond to compounds such as our bicyclo-somatostatin, Wy-40,391, because of insufficient binding. Substitution of the L-tryptophan residue at position 8 with D-tryptophan, Wy-41,524, tends to restore the full biological spectrum (Table II,

exp. E) consistent with the finding that such a substitution for somatostatin causes an eight-fold increase in potency (12). The D-tryptophan moiety seems to amplify functions which are otherwise latent or weak, perhaps through increasing the affinity to the receptors.

The remarkable specificity of Wy-40,391 could be beneficial for the treatment of hypersecretion of growth hormone in humans.

REFERENCES

1. Brazeau, P., Vale, W., Burgus, R., Ling N., Butcher, M., Rivier, J., and Guillemin, R. (1973) Science 179, 77-79.
2. Alford, F.P., Bloom, S.R., Nabarro, J.D.N., Hall, R., Besser, G.M., Coy, D.H., Kastin, A.J., and Schally, A.V. (1974) Lancet ii, 974-976.
3. Alberti, K.G.M.M., Christensen, S.E., Iversen, J., Seyer-Hansen, K., Christensen, N.J., Hansen, A.A.P., Lundbaek, K., and Orskov, H. (1973) Lancet i, 1299-1301.
4. Bloom, S.R., Mortimer, C.H., Thorner, M.O., Besser, G.M., Hall, R., Gomez-Pan, A., Roy, V.M., Russell, R.C.G., Coy, D.H., Kastin, A.J., and Schally, A.V. (1974) Lancet ii, 1106-1109.
5. Boden, G., Sivitz, M.C., Owen, O.E., Konmar, N.E., and Landor, J.H., (1975) Science 190, 163-164.
6. Sarantakis, D., McKinley, W.A., Jaunakais, I., Clark, D., and Grant, N.H. (1976) Clinical Endocr., 5s. 275-278.
7. Grant, N., Clark, D., Garsky, V., Jaunakais, I., McGregor, W., and Sarantakis, D. (1976) Life Sciences 19, 629-632
8. Gisin, B.F. (1973) Helv. Chim. Acta., 56, 1476-1482.
9. Konig, W. and Geiger, R. (1972) Chemistry and Biology of Peptides, 3rd American Peptide Symposium, Boston, Ann Arbor Science Publisher, Ann Arbor, Michigan, 343.
10. Honzl, J. and Rudinger, J. (1961), Coll. Czech. Chem. Commun. 26, 2333-2344.
11. Brazeau, P., Rivier, J., Vale, W. and Guillemin, R. (1974) Endocrinology 94, 184-187.
12. Rivier, J., Brown, M., and Vale, W. (1975) Biochem. Biophys. Res. Commun., 65 746-751.