D-Trp^{8}-Somatostatin: An analog of somatostatin more potent than the native molecule.

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SUMMARY: D-Trp^{8}-somatostatin (Ala-Gly-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH) was synthesized by solid phase methodology. This tetradecapeptide was found to be 8 times more potent than somatostatin under different assay conditions. This finding is interpreted in terms of conformational aspects of somatostatin or of enzymic resistance to degradation.

We recently reported the synthesis of a series of short chain analogs of somatostatin (Rivier et al., 1975a; Brazeau et al.,1974) which consisted essentially of the ring structure of somatostatin or acylation thereof and also des-Ala^{1}-Gly^{2}-desamino-Cys^{3}-somatostatin. On the basis of the high potency of these analogs, we concluded that the 38 membered ring contains all the necessary information for receptor recognition. Since then we have systematically substituted each amino acid by an L-alanine residue (Vale et al., 1975a) and found that, with the exception of Ala^{2}- and Ala^{5}-somatostatin, most substituted analogs had low but significant potencies. Ala^{8}-somatostatin however, had less than 0.1% potency. The high potency of Ala^{2}- and D-Ala^{2}-somatostatin (Rivier et al., in preparation) is one more indication that the side chain conformation is of little importance. The high potency of Ala^{5}-somatostatin is more difficult to explain and leads one to believe that the Asn^{5} side chain is only there to contribute to the overall hydrophilicity of somatostatin or, as suggested by Holladay and Puett's proposed tertiary conformation (1975), to contribute an extra hydrogen bond in between the carboxamide N-H and the carbonyl of Thr^{12} for additional stabilization. Holladay and Puett's proposed tertiary structure for somatostatin based on circular dichroic studies, favor a hairpin turn between residues 5 and 11 and a β- structure with peptide hydrogen bonding between residues 5-11 and 7-9. Additional stabilization by stacking of the three aromatic rings, Phe^{7}, Trp^{8} and Phe^{11} is also proposed. Predictions by Chou and Fasman (1974) favor such a β sheet conformation for that particular amino acid sequence from residue 6 to 12 even though, according to

Kabat and Wu (1973), Phe^7, Trp^8 and Lys^9 are β sheet breaking residues in the somatostatin sequence. It was thus of interest to synthesize D-Trp^8-somatostatin which would have an altered backbone conformation in that area.

MATERIALS AND METHODS

Synthesis and characterization: D-Trp^8-somatostatin was synthesized by solid phase methodology (Rivier et al., 1974, 1975a) on a chloromethylated resin (SX-1: 0.75 mEq Cl/g Lab System). Both D and L protected amino acids used in the synthesis were purchased from Bachem Inc. α-tBoc L- and D-tryptophan had both the same absolute specific optical rotation. Boc-SpOMe-Bzl-Cys was linked to the polymer by a modification of Monahan and Gilon procedure (1973). Only one mEq of protected amino acid per mEq of Cl on the resin was used. To the slurry of the resin and the dissolved protected amino acid in DMSO is added 0.9 mEq of KOtBut (as a solid fluffy white powder from Matheson) per mEq of amino acid derivatives. The reaction mixture is exposed to air as little as possible so that one does not observe any amber coloration of the solution. Reaction at 80°C for 2 hrs yield suitably subsituted resin for peptide synthesis (0.2-0.4 mEq/g of resin). Caution: a) the protected amino acids should be completely dissolved before the addition of the base (heating might be necessary). b) complete dissolution of the base should be obtained by appropriate shaking of the reaction slurry.

The building of the peptide chain on the resin was obtained using previously described procedures (Rivier et al., 1975a) with the following modifications: α-Boc-Lys-(ε-2ClZ) (Erickson and Merrifield, 1973) was used for residue 9, Boc-D-Trp for residue 8.

The protected peptide resin (5 g) was treated with HF (75 ml) for 45 minutes at 0°C in the presence of anisole (8 ml). After rapid removal of HF and drying under vacuum, the off-white colored resin was washed with ether (4 x 50 ml). The dried resin was immediately extracted with 25% AcOH (150 ml) and diluted to 3000 ml with degassed $H_2O(N_2)$. The pH of the solution was adjusted to 6.6 with NH_4OH. The solution was titrated dropwise under stirring with potassium ferricyanide solution (1 g/500 ml H_2O) until a permanent yellow color was observed. The solution sat for 10 minutes and the pH was adjusted to 5.0 with glacial AcOH; Bio Rad AG 3-X4A (100-200 mesh, chloride form, 10-15 g) was added to the turbid solution and stirred for 15 minutes. The solution was filtered over celite and applied successively onto two columns; a) Bio Rad AG 3-X4A chloride form (10 ml); b) Bio Rex-70 (70 ml) cation form. The celite + resin cake was thoroughly washed with water (500 ml) which was

applied onto columns a) and b) as a wash. The peptide material was then eluted from the Bio Rex-70 column with pyridine:acetic acid:water (30:4:66) (Hope et al., 1962). Fractions were collected; only the ones containing peptide (ninhydrin positive) were diluted with water and immediately lyophilized. 1.2 g of crude cream colored material was obtained. It was applied onto a Sephadex G-25 F gel column (3 x 200 cm) equilibrated and eluted with 2 N AcOH. The elution pattern as observed at 280 nm showed one major symmetrical peak centered at 2 V_0 (260 mg). It was subsequently applied onto a partition column 1.8 x 100 cm (n-butanol:acetic acid:water, 4:1:5)(BAW). The elution pattern (280 nm) showed one major peak from V_0 2.5-4.0. Two cuts were made which subsequently appeared to be identical on tlc (165 mg)(approximately 80% pure). This material (150 mg) was applied in 0.1 M NH_4OAc pH 7.0 onto a CMC column (4.5 x 1 cm; 5 ml) and eluted with 0.2 M NH_4OAc pH 7.0. In our hands, purification on CMC eliminates the tailing impurities so often observed on tlc for synthetic peptides. The desired product eluted as a very narrow band which after 2 lyophylizations gave a white fluffy powder (115 mg). This zone however, still showed a minor (less than 5%) impurity running in front of D-Trp^8-somatostatin when applied on silica gel plates (Eastman 6061) and run with the upper phase of a BAW system. This material (100 mg) was applied on a partition column (identical to that in step 2). The elution pattern (280 nm) showed one peak centered at 3.3 V_0. 65 mg of homogeneous material was obtained after lyophylization (see table I for physical constants). The specific optical rotation $[\alpha]_D^{23}$ = -47.3 ± 0.5 (c = 1 in 1% AcOH) is quite different from that of somatostatin $[\alpha]_D^{23}$ = -33.3 ± 0.5 under the same conditions. Amino acid analysis under the conditions previously reported (Rivier et al., 1975a) indicates the following ratios: Lys, 1.95; NH_3, 1.50; Trp, 0.88; Asp, 1.00; cysteic acid, 1.72; Ser, 0.81; Thr, 1.81; Gly, 0.97; Ala, 0.98; Phe, 2.94.

Biological testing:

The in vitro biological assay (Vale et al., 1972; Vale and Grant, 1975b) is based on the ability of somatostatin and its analogs to inhibit the spontaneous secretion of radioimmunoassayable growth hormone by primary cultures of enzymatically dissociated rat anterior pituitary cells. This test is highly quantitative as well as specific since it has been used to follow somatostatin throughout its isolation (Brazeau et al., 1973a; Vale et al., 1975a). The in vivo biological assay (Brown et al., 1975) is based on somatostatin or somatostatin analog inhibition of insulin and glucagon release induced by arginine in the rat.

BIOLOGICAL RESULTS AND DISCUSSSION

The enhanced biological potency of D-Trp^8-somatostatin as compared to that of somatostatin is shown in Table II.

TABLE I: Rf Values of Somatostatin and D-Trp^8-Somatostatin [1]

	Solvent System [2]					Electro-phoresis [3]
	(I)	(II)	(III)	(IV)	(V)	
Somatostatin	.44	.10	.39	.78	.41	.34
D-Trp^8-Somatostatin	.42	.10	.38	.76	.37	.34

1) Rf values were determined on Eastman No. 6061 silica gel sheets. The spots were detected by I_2 and ninhydrin spray. Loads varied from 20-40 µg per spot. The solvent front ran at least 12 cm.

2) Solvent systems:
I) 1-BuOH:pyridine:0.1% HOAc (5:3:11)
II) 1-BuOH:2-PrOH:1N NH_4OH:EtOAc (1:1:2.5:1)
III) 1-BuOH:HOAc:H_2O (4:1:5)
IV) i-PrOH:1N AcOH (1:1)
V) i-AmylOH:pyridine:H_2O (7:7:6)
For the two phases solvent systems, only the upper phase was used.

3) Rf values relative to lysine and alanine, 100 µg loads on Whatman 3MM (pH 4.7 buffer containing 2.5% acetic acid, 2.5% pyridine, 5% 1-BuOH, and 90% water) at 3500 V. 2.0-hr run.

If it were not for the high biological potency of this compound, one might question the optical purity of the D-Trp^8 residue. In view of the optical purity of the starting tBoc-D-Trp and also because racemisation has only rarely been observed in solid phase synthesis (Meienhoffer, 1973), we have not investigated the optical purity of the Trp residue once incorporated in the molecule. However, somatostatin and D-Trp^8-somatostatin have different Rf in systems I and V, and an equal load of a mixture shows unequivocally two spots in the latter system. On the basis of that observation, we can rule out the presence of as low as 1% of somatostatin in our preparation of D-Trp^8-somatostatin and vice-versa. That our preparation of D-Trp^8-somatostatin is free of somatostatin has also been shown by radioimmunoassay (Vale, unpublished results). The fact that substitution of Trp by D-Trp at the 8 position in this tetradecapeptide yields two compounds with different Rf on tlc in more than one system, is quite unusual and must reflect dramatic changes in the overall contour of the molecule.

In summary, D-Trp^8-somatostatin is 6-8 times more potent than somatostatin to inhibit the release of growth hormone, glucagon and insulin under

TABLE II. Relative Potencies of Somatostatin and D-Trp^8-Somatostatin

	In vitro GH	In vivo Glucagon	In vivo Insulin
Somatostatin	100	100	100
D-Trp^8-somatostatin	848(518-1416)[a]	639(205-1665)[a]	821(368-2195)[a]

a) 95% confidence limits are shown in parenthesis.

the conditions reported. This is the first analog to be reported to have significantly higher potency than somatostatin. It is of interest to note that the increased specific activity of D-Trp^8-somatostatin when related to somatostatin as the reference standard, is the same on three different receptor sites, i.e., the pituitary for release of growth hormone, and the endocrine pancreas for the release of glucagon and insulin. This can be the reflection of a stabilized tertiary structure in a conformation favorable for receptor-interaction, or an increased resistance to degradation by biological fluids. D amino acids are known to resist specific enzymatic degradations (Marks and Stern, 1974): for example, D amino acids replacing the glycine 6 residue in the LRF molecule (Monahan, 1973) had yielded compounds with prolonged action in vivo and up to 15 times more active than the parent hormone (Vale et al., 1975b,c; Rivier et al., 1975b). Both the functionality of the side chain and the stabilization of the backbone conformation by introduction of a D configuration can be proposed to explain such a drastic effect on the active conformation unless such structural modifications influence the clearance rate (in vivo) and/or the kinetics or mechanism of enzymatic degradation (in vivo or in vitro). Studies are presently pursued to clarify these points. The immediate advantage of such a structure is its lower cost when compared with that of somatostatin for a given active dose.

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