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Somatostatin Analogs. Relative Importance of the Disulfide Bridge and of the Ala-Gly Side Chain for Biological Activity

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Four analogs of ovine somatostatin (SRIF, somatotropin release inhibiting factor), the sequence of which is H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH, have been synthesized by the solid-phase methodology. The compounds were assayed and were found to possess the following somatotropin release inhibiting potencies relative to pure synthetic somatostatin *in vitro* and *in vivo*, respectively: [$Ala^{3,14}$]somatostatin, 0.6 and 2.0%; [$SMe\text{-}Cys^{3,14}$]somatostatin, 4 and 0.6%; [$NAc\text{-}Cys^{3}$]somatostatin, 39 and 105%; [$des\text{-}Ala^{1}\text{-}Gly^{2}$]somatostatin, 65 and 71%. The dihydrosomatostatin analogs [$NAc\text{-}Cys^{3}\text{-}H_2$]somatostatin and [$des\text{-}Ala^{1}\text{-}Gly^{2}\text{-}H_2$]somatostatin after two purifications by gel filtration were assessed to be at least 80% homogeneous and had respectively 99 and 89% of somatostatin potencies *in vivo*. Structure–activity relationships are discussed.

We recently reported the structure of the first hypothalamic release inhibiting factor which controls the secretion of somatotropin.[1-3] Somatostatin or somatotropin release inhibiting factor (SRIF) of ovine origin is an heterodetic cyclic homomeric tetradecapeptide, the primary structure of which is H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH.[4,5] A synthetic replica in its reduced (dihydrosomatostatin) or oxidized form (somatostatin) was found to exhibit, qualitatively and quantitatively, the same biological properties[6,7] *in vitro* and *in vivo* under certain circumstances[8] as that of the natural hormone. Investigations on the biological properties of somatostatin have been reported[9-13] and extensive clinical studies with either form of the peptide have already appeared in the literature.[14-19]

In an attempt to better understand the role of the disulfide bridge and the role of the Ala-Gly side chain in the molecule, we synthesized two analogs in which $Cys^{3,14}$ has been replaced by Ala or the sulfhydryls have been alkylated, and two shorter analogs where $Ala^{1}\text{-}Gly^{2}$ has been deleted or replaced by an acetyl group. A preliminary report of these data appeared in ref 9.

Synthesis, Purification, and Characterization. [$SMe\text{-}Cys^{3,14}$]-SRIF (**2**) and [$Ala^{3,14}$]-SRIF (**3**) were synthesized by the solid-phase method previously described[7] using Boc-Cys(SMe) and Boc-Ala at the 3 and 14 positions. [$Des\text{-}Ala^{1}\text{-}Gly^{2}$]-SRIF (**4** and **5**) and [$Ac\text{-}Cys^{3}$]-SRIF (**6** and **7**) were obtained from an SRIF resin lacking the two amino acids alanine and glycine. Acetylation was done on the resin after deprotection of the cysteine residue at the 3 position using an excess of acetic anhydride.

The dihydrosomatostatin analogs **4** and **6** were obtained according to the reported procedure[7] with a few improvements, as described in the Experimental Section. The cyclic form **5** or **7** was obtained after ferricyanide oxidation according to du Vigneaud.[20] Purification consisted of ion-exchange chromatography on carboxymethylcellulose and partition chromatography on Sephadex G-25F as previously described.[7] Two successive gel filtrations in 2 *M* AcOH and 10^{-2} *M* β-mercaptoethanol on Sephadex G-25F were performed to purify **4** and **6**.

After purification, these peptides were characterized by amino acid analysis and their specific optical rotation (Table II). These new compounds were homogeneous in six thin-layer chromotography (on silica gel) systems and electrophoresis on paper (see Table II) with the exception of analogs **4** and **6** for which we have not found any suitable thin-layer system. In these cases purity can be assessed to be better than 80% on the basis essentially of the amino acid analyses. Assessment of purity by countercurrent distribution is discussed in the Experimental Section.

Biological Activity. *In vitro*, the peptides were assayed for their ability to inhibit the spontaneous secretion of radioimmunoassayable growth hormone by rat anterior pituitary cells placed in culture according to Vale, *et al.*[21] Each peptide was assayed three times or more. *In vivo* the peptides were assayed for their ability to inhibit the elevation of plasma growth hormone concentration induced in rats (six per point) by acute administration of sodium pentobarbital 15 min before decapitation. See Brazeau, *et al.*,[8,11] for experimental details and Table I for specific biological activities; each peptide was tested *in vivo* twice or more. For both *in vitro* and *in vivo* experiments the peptides were studied in complete six-point assay designs with somatostatin as a reference standard.

Our first observation that [H_2]somatostatin had the same biological potency as somatostatin[1] led us to speculate about the relative importance of the disulfide bridge which would be expected to stabilize the tertiary structure of somatostatin unless strong interactions, such as ionic, hydrophobic, dipolar, etc., within the molecule were already maintaining the molecule in a shape readily recognizable by the receptor.

Table I. Specific Biological Activity of Somatostatin Analogs

No.	Compd	% specific activity of somatostatin (95% confidence limits) *In vitro*	*In vivo*[a]
1	Somatostatin	100	100
2	[SMe-Cys^{3,14}]-Somatostatin	4.0 (2.0–6.0)	0.6 (0.25–1.05)
3	[Ala^{3,14}]-Somatostatin	0.6 (0.32–0.95)	2.0 (0.80–3.4)
4	[Des-Ala^1-Gly^2-H$_2$]-Somatostatin	33 (20–50)	89 (72–110)
5	[Des-Ala^1-Gly^2]-Somatostatin	65 (42–98)	71 (57–88)
6	[NAc-Cys^3-H$_2$]-Somatostatin	30 (18–47)	99 (80–122)
7	[NAc-Cys^3]-Somatostatin	39 (25–60)	105 (86–130)

[a] All peptides were administered intravenously.

To test that hypothesis we synthesized 2 and 3. Their low but significant biological activity *in vitro* (1 and 0.1%, respectively) would suggest that only a small portion of these analogs exists in a favorable shape for receptor recognition and, hence, the disulfide bridge is important for activity. Since these analogs differ from somatostatin in additional respects other than being unable to cyclize (lack of sulfhydryl groups in the case of [Ala^{3,14}]somatostatin and possible steric hindrance for [SMe-Cys^{3,14}]somatostatin) alternative explanations cannot be ruled out. Concomitantly, with a preliminary communication of the biological activity of 2 and 3,[9] a report by Sarantakis, *et al.*,[22] reached the same conclusion on the basis of the biological activity of 3 only. We cannot, however, explain either the lower biological activity (20 times less than we observe) reported by Sarantakis, *et al.*, for 3 using the same biological assay as we described or the lower specific rotation (−44.1). Low values of threonine and maybe serine in their preparation of 3 may be considered as a reason for the observed discrepancies.

To account for the high activity of [H$_2$]somatostatin, one possibility is that somatostatin is the most potent form of the peptide and that [H$_2$]somatostatin is rapidly oxidized under the conditions of the bioassays. Our observation that somatostatin is more potent than [H$_2$]somatostatin *in vivo* when injected subcutaneously[11] at the time of maximum stimulation by sodium pentobarbital is not necessarily in favor of this hypothesis. Indeed, under those assay conditions, diffusion rate might be the limiting factor as evidenced by the observed prolonged action of [H$_2$]somatostatin and of several other analogs where diffusion is controlled by lowering their solubility, *i.e.*, 6 and 7.[23]

It is thus evident that the relative potency of the different analogs described will be very dependent upon the assay system under which they were tested. *In vitro* systems will give an integrated value whereas *in vivo* data will be able to show the dynamics of the inhibition. We will, therefore, not try to speculate or to reconcile results obtained independently *in vitro* and *in vivo*. The high biological activities *in vivo* and *in vitro* of 4 and 6 and of 5 and 7 are of great interest since it points out that the 38-membered ring contains all the information necessary for recognition by the pituitary receptors. In our search for the smallest active fragment we also synthesized [des-Ala^1-Gly^2-desamino-Cys^3]somatostatin which was found to have *in vitro* 60% of somatostatin potency (Rivier, *et al.*, in preparation). It is known that somatostatin acts directly on the pancreas to inhibit the release of glucagon and insulin.[12] Experiments are being carried out to see if the Ala^1-Gly^2 side chain is of any specific importance for recognition by the pancreatic receptors. Dissociation of biological activities could be of great therapeutic value in the treatment of acromegaly and diabetes.[14-19]

Experimental Section

Homogeneity of the peptides 2, 3, 5, and 7 was demonstrated by thin-layer chromatography on Eastman chromatogram sheets (6061 silica gel with fluorescent indicator) in six solvent systems; see Table II.

Amino acid analyses were performed on peptide hydrolysates (6 *N* HCl + 2.5% thioglycolic acid at 100° in evacuated sealed tubes for 20 hr) using a Beckman/Spinco Model 119 amino acid analyzer. No correction was made for decomposition of Trp, Cys and Ser values were obtained after performic oxidation as reported in ref 7. Peak areas were determined by an Infotronics Model CRS-100A electronic integrator. The starting materials† were bought from Bachem and were found to be homogeneous and optically pure by determination of their optical rotation. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter. All purifications by partition chromatography were done on Sephadex G-25F using the two-phase system (*n*-BuOH–AcOH–H$_2$O, 4:1:5).

Syntheses.[7] Polystyrene cross-linked with 1% divinylbenzene resin (Bio-Rad SX-1) was chloromethylated according to standard procedures;[24] 0.8 mequiv of Cl/g of resin was obtained. Esterification was performed as described by Stewart and Young;[24] 0.15 mequiv of α-Boc-Cys(SMe), α-Boc-Cys(SpOMe-Bzl), or α-Boc-Ala/g of resin was obtained. Deblocking was achieved in 20 min by TFA–CH$_2$Cl$_2$ (50:50), followed by neutralization with 15% Et$_3$N in DMF. Successive coupling of each amino acid (5 *M* excess) was mediated by dicyclohexylcarbodiimide (5 *M* excess) for an average of 2 hr in CH$_2$Cl$_2$ with the exception of Boc-Trp which was coupled in CH$_2$Cl$_2$–DMF (1:1) and Asn introduced as its *p*-nitrophenyl ester in DMF overnight.

The reaction was controlled by the ninhydrin test of Kaiser, *et al.*[25] Washes included DMF, CH$_2$Cl$_2$, and MeOH. Acetylation in the case of compounds 6 and 7 was done on the resin using (AcO)$_2$O (50 *M* excess) in CH$_2$Cl$_2$ for 0.5 hr. A 0.75 hr HF treatment[26] at 0° in the presence of anisole deblocked and cleaved the peptide resin (3 g). HF was thoroughly eliminated under high vacuum. The crude peptides mixed with the resin were washed with anhydrous ether and extracted with 0.2 *N* AcOH.

[SMe-Cys^{3,14}]Somatostatin (2). After HF treatment, ether wash, and extraction with 0.2 *N* AcOH (200 ml), the solution was lyophylized yielding a crude cream-colored fluffy material (500 mg). This material (300 mg) was applied on a column (9 × 2.5 cm) for ion-exchange chromatography (carboxymethylcellulose; linear concentration gradient 0.05–0.5 *M* NH$_4$OAc, pH 7, mixing vessels 300 ml). Observation at 280 nm showed a major peak appearing when the concentration of the buffer reached 0.2 *M*. Two lyophilizations yielded 200 mg of a white powder. Further purification on a partition column (100 × 1.6 cm, applied 170 mg) yielded the highly purified material (75 mg) in a peak (280 nm) centered at 3.2 V_0. Amino acid composition: Ala (1.01), Gly (0.99), Lys (1.88), Asp (1.00), Phe (2.94), Trp (0.77), Thr (1.80), Ser (0.72).

[Ala^{3,14}]Somatostatin (3). After HF treatment, ether wash, and extraction with 0.2 *N* AcOH (200 ml), the solution was lyophylized yielding a crude white fluffy material (550 mg). This material (400 mg) was applied on a column (100 × 1.8 cm) for partition chromatography. Observation at 280 nm showed a major peak at 3.8 V_0 (130 mg). A second pass yielded the highly purified material (85 mg). Amino acid composition: Ala (3.00), Gly (1.02), Lys (1.90), Asp (1.00), Phe (2.91), Trp (0.54), Thr (1.98), Ser (0.89).

[Des-Ala^1-Gly^2]Dihydrosomatostatin (4) and [Ac-Cys^3]Dihydrosomatostatin (6). Improved Approach for the Purification of Dihydrosomatostatin and Dihydrosomatostatin Analogs.

†Starting protected amino acids: α-Boc-Cys(pOMe-Bzl); α-Boc-Ser(OBzl); α-Boc-Thr(OBzl); α-Boc-Trp; α-Boc-Phe; α-Boc-Lys(ε-Z); α-Boc-Asn-PNP; Z-Ala-Gly; α-Boc-Cys(SMe); α-Boc-Ala.

Table II. Physical Constants and Yield of Somatostatin Analogs[e]

Compd	Tlc system[a] BPA	EPAW	IaPW	IpA	BAW	BIpNE	Electrophoresis[b]	$[\alpha]^{20}D$,[c] deg	Yield,[d] %
2	0.43	0.49	0.51	0.72	0.30	0.15	0.34	−39.4	11
3	0.30	0.43	0.36	0.60	0.27	0.11	0.36	−47.5	13
5	0.53	0.52	0.60	0.80	0.37	0.18	0.36	−41.5	4
7	0.60	0.57	0.65	0.85	0.44	0.21	0.15	−34.7	8

[a]BPA, 1-butanol–pyridine–0.1 *N* acetic acid (5:3:11, upper phase); EPAW, ethyl acetate–pyridine–acetic acid–water (5:5:1:3); IaPW, isoamyl alcohol–pyridine–water (7:7:6); IpA, 2-propanol–1 *N* acetic acid (2:1); BAW, 1-butanol–acetic acid–water (4:1:5, upper phase); 1-butanol–2-propanol–1 *N* ammonia–ethyl acetate (1:1:1:2.5). Uv, I_2, and ninhydrin spray were successively used. Loads varied from 20 to 40 μg per spot. [b]R_f 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-butanol, and 90% water) at 3500 V, 2.0-hr run. [c]Concentration in 1% AcOH = 1. [d]Peptide yields are calculated on the basis of millimoles of peptides isolated after final purification relative to the total millimoles of starting *tert*-butyloxycarbonylamino acid, *viz.*, as resin ester. In the case of the cyclic compounds **5** and **7** yields were not optimized. [e]The purity of compounds **4** and **6** is hard to assess due to numerous artifacts (essentially due to the free sulfhydryls) observed on the tlc support used. Our estimate is better than 80%.

After HF treatment and ether wash, the resin was extracted with 2 *N* AcOH (10 ml). The syrupy solution was directly applied on a column of Sephadex G-25F (2.5 × 200 cm) for gel filtration (eluent 2 *N* AcOH, 10^{-2} *M* β-mercaptoethanol). Observation at 280 nm showed a major peak at 2.0 V_0 which after lyophilization yielded a white material (450 mg, 70%). A second pass on the same column generally shows a fairly symmetrical pattern with yields varying from 200 mg (32%) to 300 mg (48%) of purified material that we estimate to have less than 20% contaminants (failure sequences, chain termination, etc.). By directly applying the crude mixture obtained after HF cleavage onto a gel column, we could avoid aggregation problems often observed when we tried to redissolve the lyophylized crude mixture, thus considerably improving yields. An attempt to further purify the dihydrosomatostatin analog **6** by countercurrent distribution in the presence of a strong reducing agent (dithiothreitol) failed in the sense that extensive tailing was observed. The partition system used was composed of *n*-BuOH–AcOH–H_2O (4:1:5). The partition coefficient is close to 1 (0.96). One hundred transfers were performed. A major symmetrical peak is observed coming out of a high background (60% of the weight being recovered in the main peak). The main peak was rerun in the same system after lyophylization; it showed a profile similar to that of the former run proving that no purification had been performed. This behavior is not unusual and can probably be best explained by nonspecific interactions, the concentration of peptide being in the order of 10 mg/20 ml at the peak tube. Quantitative determination of sulfhydryl contents ranged in different preparations from 60 to 80% of the theoretical value.[7]

[Des-Ala[1]-Gly[2]]Somatostatin (5) and [NAc-Cys[3]]Somatostatin (7). General Approach for the Synthesis of Somatostatin and Somatostatin Analogs (Potassium Ferricyanide Oxidation). Batches of protected peptide resin (5 g) were treated with HF (75 ml) for 45 min at 0° in the presence of anisole (6 ml). After removal of HF and drying *in vacuo*, the resin was washed with ether (300 ml). The dried resin was immediately extracted with 2 *N* AcOH (500 ml) and diluted with 4500 ml of H_2O. The pH of the solution was adjusted to 6.6–7.5 with NH_4OH. The solution was titrated dropwise under stirring with potassium ferricyanide solution (0.005 *N*) until a permanent yellow color was observed (250–350 ml). A small excess (5–10%) of ferricyanide solution was added to ensure complete oxidation and the solution stirred for an additional 10 min. After adjustment of the pH to 5 with glacial AcOH, approximately 100–200 ml of Bio-Rad AG3-4A (100–200 mesh, chloride form) was added to the turbid solution and stirred 15 min. The resin was filtered off in the form of a column and the filtrate passed through twice to remove ferricyanide and ferrocyanide ions. Bio-Rex 70 (150 ml, cation form) was then added and stirred 20 min. The resin was filtered off in the form of a column. The filtrate was passed over the resin three additional times to ensure complete adsorption of the peptide. The peptide material was then eluted with pyridine–acetic acid–water (30:4:66) and concentrated to a small volume and lyophilized.

The cyclized peptide was then desalted on a Sephadex G-10 column (5 × 50 cm) with 0.5 *N* AcOH. The major excluded peak and its tailing end were lyophilized. This material (800 mg) was applied on a column (9 × 2.5 cm) for ion-exchange chromatography (carboxymethylcellulose; linear concentration gradient 0.05–0.5 NH_4OAc, pH 7, mixing vessels 300 ml). Observation at 280 nm showed a major peak appearing when the concentration of the buffer reached 0.2 *M*. Two lyophilizations yielded 300 mg of a white powder. Further purification on a partition column (100 × 1.6 cm, applied total amount) yielded the highly purified materials after 3 V_0 in the case of somatostatin. For somatostatin we consistently obtained a yield of 80–100 mg from 5 g of peptide resin.

Starting with 3 g of peptide-resin, in the case of [des-Ala[1]-Gly[2]]-somatostatin (**5**) we obtained after the partition column (60 × 1.2 cm) 20 mg of a highly purified material eluting at 4 V_0. Amino acid composition: Cys (1.62), Lys (1.86), Asp (1.00), Phe (2.85), Trp (0.86), Thr (1.84), Ser (0.85). Starting with 3 g of peptide-resin, in the case of [NAc-Cys[3]]somatostatin (**7**), we obtained after partition chromatography (100 × 1.6 cm) 40 mg of highly purified material eluting at 2.5 V_0. Amino acid composition: Cys (1.70), Lys (2.10), Asp (1.00), Phe (2.94), Try (0.68), Thr (1.96), Ser (0.94). In all these cases no emphasis was put on yields.

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Distal Conformation of Thyroid Hormones. Crystal and Molecular Structure of 3,5,3′-Triiodo-L-thyronine Methyl Ester

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In the crystal structure of 3,5,3′-triiodo-L-thyronine methyl ester, the 3′-iodine is distal, *i.e.*, away from the alanine bearing ring, and the overall conformation is cisoid, that is, the alanine moiety and the outer phenyl ring lie on the same side of the inner phenyl ring plane. This conformation, reported here for the first time, is in contrast to the transoid conformation previously observed for thyroid hormone structures. The torsional angles between the diphenyl ether linkages (ϕ and ϕ') are −108 and 33°, respectively, while the C–O–C angle is 117°. The value of χ^1, which describes the amino acid backbone conformation, is 308°. The structure crystallizes in the tetragonal space group $P4_1$ with a = 8.225 (5) and c = 28.42 (1) Å. The final R index is 0.06.

Recently extensive studies of the molecular conformation of thyroid hormones and their analogs have been made in an effort to understand the structural requirements for their biological activity. There is now an accumulation of evidence which suggests that the hormone 3,5,3′-triiodo-L-thyronine (T_3) plays a more significant role in biological activity than had been previously supposed. Recent studies have also established that thyroxine (T_4) is converted to T_3 in peripheral tissues.[1-4] There are two conformers which can result from the deiodination of thyroxine (Figure 1), *i.e.*, a distal or proximal conformer depending upon whether the 3′-iodine is away from or toward the inner phenyl ring. The results of several stereochemical, binding, and biological activity studies[5-8] strongly suggest that hormonal activity is greater for the distal orientation of the 3′-iodine.

Based on steric considerations, it had been suggested that a large barrier to internal rotation about the diphenyl ether linkage prevented easy conversion from one conformer to the other. However, recent energy calculations using modified CNDO/2 procedures[9] and those using the extended Hückel approach[10] both suggested that the energy barrier to internal rotation about the diphenyl ether linkage is relatively small (11 and 50 kcal, respectively). Neither study showed any significant preference for the distal or proximal conformer. The repeated crystallographic observation of both the distal[11,12] and proximal[13,14] 3′-iodine in triiodothyronines suggests that the two conformers are indeed stable and readily accessible in solution. We report here the first complete structural details of a thyroid hormone analog having an overall cisoid conformation with a distal 3′-iodine in the crystal and molecular structure of 3,5,3′-triiodo-L-thyronine methyl ester. This work is part of a larger program to study the molecular conformation of a series of thyroid hormones, hormone precursors, and thyroxine analogs.

Experimental Section

Crystals of 3,5,3′-triiodo-L-thyronine methyl ester were prepared by methylation of T_3 and then removal of the HCl by extraction through a column of Fisher Rexyn 203(OH). Crystals were grown from methanol solutions. A clear, well-formed crystal (0.10 × 0.18 × 0.20 mm³) was selected for intensity data collection. All X-ray measurements were made on a GE-XRD-5 single crystal counter diffractometer. The crystal was mounted with the b axis parallel to the ϕ axis of the instrument. The systematic extinctions indicated the tetragonal space group $P4_1$. The unit cell dimensions were obtained from a least-squares calculation based on 2θ measurements of 34 hkl reflections having $2\theta > 40°$, using the Mo Kα radiation for data collection. The crystal data are presented in Table I.

The intensities of 1758 (1474 observed) independent reflections with 2θ less than 50° were measured by the stationary crystal-stationary counter technique. The background was a uniform function of 2θ above 20° and a background correction curve was constructed from zirconium-yttrium balanced filter measurements of the data in this range. Reflections were considered unobserved if the net count was less than twice the standard deviation of the background. Yttrium filtered background measurements were made for all data with 2θ less than 20°. No significant changes were observed in the intensities of the standard reflections measured daily during data collection. The shape anisotropy measurements at χ = 90° were found to be less than ±3% over the ϕ range covered in the intensity collection. Intensities were corrected for Lorentz and polarization factors, and an absorption correction based on the intensity variation of ϕ at χ = 90° was applied.

The structure was solved by straightforward application of heavy atom techniques. Atomic scattering factors for iodine were taken from Cromer[15] while those for the other atoms were taken from the International Tables for X-Ray Crystallography.[16] The positional parameters and anisotropic thermal parameters for all nonhydrogen atoms were refined by using a block diagonal ap-