Highly Active Position Eight Analogues of Somatostatin and Separation of Peptide Diastereomers by Partition Chromatography†

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ABSTRACT: Six stereochemically pure analogues of somatostatin (SS), D- and L-5F-Trp^8-SS, D- and L-6F-Trp^8-SS, and D- and L-5Br-Trp^8-SS, were synthesized and found to be more potent than somatostatin in suppressing the release of growth hormone from cultured rat pituitary cells. Two of the analogues, D-5F-Trp^8- and D-5Br-Trp^8-SS, were respectively 25 and 30 times more active than somatostatin in that assay. The analogues were prepared by solid phase synthesis of their corresponding diastereomeric mixtures, followed by their complete resolution by preparative partition chromatography. Reversed phase high pressure liquid chromatography (HPLC) was used to monitor the resolution and also to check the final purity of each peptide. Positive identification of each diastereoisomer was determined by amino acid analyses of their enzymatic digests, direct comparison with a known all-L standard in the case of the 5F-Trp^8 analogues, and chromatographic separation of dansylated amino acids following enzymatic digestion of D- and L-5Br-Trp^8-SS. The role of tryptophan in somatostatin is discussed and it is suggested that maintenance of physiological activity in somatostatin peptides, at least on the pituitary, is partially dependent upon the degree of resonance in the indole nucleus in position 8.

Studies with numerous analogues of the somatostatin tetradecapeptide (Figure 1) have shown that tryptophan in position 8 is among the most important residues for expression of biological activity in several tissues. Thus, replacement of Trp with simple aliphatic or aromatic amino acid residues produced analogues with very low growth hormone release-inhibiting activity (Brown et al., 1976; Rivier et al., 1976). Rivier et al. (1975) reported and we later confirmed (Coy et al., 1976) that D-Trp^8-SS[1] is five to eight times more active than somatostatin on the inhibition of growth hormone release in vitro. Somatostatin analogues containing the D-Trp^8 modification in combination with other substitution(s) have had higher and more selective biological activities (Meyers et al., 1977; Brown et al., 1977; Schally et al., 1978). These properties improve the clinical potential of somatostatin. We became interested in examining the biological effects of modifying the indole nucleus of tryptophan in position 8 of somatostatin as part of a continued search for analogues with greater therapeutic value, and also to clarify the important role of tryptophan in this hormone.

The halogenation of ring systems is frequently used in drug design since the electronic properties and wide range of atomic radii of the halogens produce a variety of mechanistic, steric, and lipophilic effects. 5F-, 6F-, and 5Br-tryptophan (Figure 2) are commercially available, and therefore convenient choices for the examination of position 8 somatostatin analogues, although all are sold as racemic mixtures. Since the D isomer of Trp in somatostatin produced a large increase in biological activity, we expected that those analogues containing modified D-Trp^8 residues might be more active than their corresponding L diastereomers. Thus, resolution of either the racemic amino acids, or of their respective diastereomeric peptides was desirable.

Preparative partition chromatography (Yamashiro, 1964) has not previously been applied to the separation of synthetic stereoisomers of somatostatin, nor has it been generally used to prepare other diastereomeric peptide derivatives.[2] Considering the differences in physical characteristics observed in previous stereoisomers of SS (Rivier et al., 1975), the comparative ease offered by separating diastereomeric peptides during routine purification procedures rather than resolving the initial amino acids, and the general utility which such a method could provide, it seemed worthwhile to attempt the preparation of stereochemically pure peptides from diastereomeric mixtures by partition chromatography. By using HPLC to monitor the degree of separation achieved, the powerful resolving capability of this method could be used to full advantage.

Materials and Methods

Amino acids are of the L configuration unless otherwise noted. All halogenated tryptophans were obtained as racemic amino acids from Aldrich Chemical Co. HPLC was performed on a Waters Associates Model 204 liquid chromatograph equipped with two Model 6000A pumps and a Model 660 gradient programmer. Amino acid analyses were run on a Beckman Model 119 equipped with a System AA computing integrator. The modified single column method employed has been described (Coy et al., 1974).

Boc-L-5-fluorotryptophan. This derivative and the resolution of the DL-amino acid used in its preparation were described previously (Coy et al., 1974).

Boc-DL-5-fluorotryptophan. This was prepared directly from the DL-amino acid by the method described previously for the pure Boc-L stereoisomer (Coy et al., 1974). The product had the same melting point (157–158 °C) as the pure L derivative, yield 80%.

Boc-DL-6-fluorotryptophan. The DL-amino acid was converted by the method of Ali et al. (1972) in 59% yield, mp

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[1] Abbreviations used: SS, somatostatin; GH, growth hormone; LH-RH, luteinizing hormone releasing hormone; Boc, *tert*-butyloxycarbonyl.

[2] While this work was in progress, Yamamoto et al. (1977) reported the resolution of labeled diastereomeric vasopressin derivatives by partition chromatography.

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TABLE I: Properties of Somatostatin Analogues.

Substituent in position 8	Yield[a] (%)	$[\alpha]_D$ (deg) in 0.1 M AcOH	R_f(I)[b]	R_f(II)	R_f(III)	R_f(IV)
L-5F-Trp[c]	11	−36 (*c* 0.50, 26 °C)	0.07	0.36	0.37	0.58
L-5F-Trp	18	−32 (*c* 0.50, 25 °C)	0.07	0.36	0.37	0.58
D-5F-Trp	12	−44 (*c* 0.50, 26 °C)	0.07	0.35	0.37	0.55
L-6F-Trp	14	−40 (*c* 0.55, 23 °C)	0.08	0.36	0.38	0.59
D-6F-Trp	11	−43 (*c* 0.52, 24 °C)	0.07	0.36	0.38	0.57
L-5Br-Trp	13	−31 (*c* 0.61, 25 °C)	0.08	036	0.38	0.59
D-5Br-Trp	9	−36 (*c* 0.56, 23 °C)[d]	0.07	0.35	0.38	0.56

[a] Yields based on final products compared with total mM of starting Boc-amino acid esterified to the resin. [b] The following TLC solvent systems were used: R_f(I), *n*-BuOH–AcOH–H_2O (4:1:5, upper phase); R_f(II), *i*-PrOH–1 M AcOH (2:1); R_f(III), *n*-BuOH–AcOH–H_2O–EtOAc (1:1:1:1); R_f(IV), EtOAc–pyridine–AcOH–H_2O (5:5:1:3). Samples (20–40 μg) were applied to Brinkman SIL-G25 plates and solvent fronts allowed to travel 10–15 cm. Spots were visualized with ninhydrin and Ehrlich's reagents. [c] Prepared from Boc-L-5F-Trp to serve as the all-L standard. [d] 50% AcOH was used as the solvent.

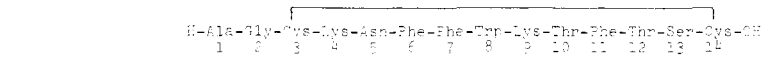

FIGURE 1: Structure of somatostatin.

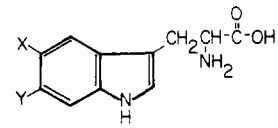

FIGURE 2: Structures of halogenated tryptophans: 5F- and 5Br-Trp (X = F or Br; Y = H); 6F-Trp (X = H; Y = F).

127–129 °C. Anal. Calcd for $C_{16}H_{19}O_4N_2F$: C, 59.62; H, 5.94; N, 8.69. Found: C, 59.80; H, 6.12; N, 8.63.

Boc-DL-5-bromotryptophan. The DL-amino acid was converted as above in 81% yield, mp 157–159 °C. Anal. Calcd for $C_{16}H_{19}O_4N_2Br$: C, 50.14; H, 5.00; N, 7.31. Found: C, 50.34; H, 5.18; N, 7.27.

Peptide Synthesis. The peptides were synthesized stepwise by the solid phase method (Merrifield, 1963) as described previously for somatostatin analogues (Meyers et al., 1977). Diisopropylcarbodiimide was used in place of dicyclohexylcarbodiimide as the coupling reagent in each cycle (Coy et al., 1977). Coupling reactions were monitored by the ninhydrin test (Kaiser et al., 1970) and repeated, if incomplete after 60 min, using the appropriate symmetric anhydride prepared as described previously (Blake & Li, 1976), but in dimethylformamide at room temperature. Any remaining free amino groups were acetylated with acetic anhydride or acetylimidazole. Protected peptides were liberated from their solid supports and deprotected by treatment with HF, then immediately cyclized in dilute aqueous solution as described previously (Meyers et al., 1977).

Purification of Peptides. Each crude diastereomeric peptide mixture was subjected to gel filtration on columns (2.5 × 95 cm) of Sephadex G-15 in 50% AcOH and Sephadex G-25 in 0.2 M AcOH. Further purification and partial separation of the diastereomeric peptides were accomplished by partition chromatography on a Sephadex G-25 column (2.5 × 95 cm) in 1-butanol:AcOH:H_2O (4:1:5 v/v). Fractions (10 mL) corresponding to the main peaks of a partially resolved doublet (300 nm) were pooled separately, concentrated in vacuo and lyophilized. The mixtures, significantly enriched in their respective diastereomeric components, and free of other contaminants as judged by HPLC, were separately passed through a Sephadex G-25 partition column (1.5 × 140 cm) in 1-butanol:2 M AcOH (1:1 v/v). In each case, the main peak (280 nm) was separated from a single smaller peak corresponding to the contaminating diastereomer with little or no overlap. HPLC of the column fractions was used as a guide to separately pool the peaks to ensure isolation of pure components. Pooled fractions were concentrated in vacuo and lyophilized. Yields and optical rotations of the six analogues are reported in Table I. Authentic L-5F-Trp[8]-SS was prepared in the usual manner (Meyers et al., 1977) using pure Boc-L-5-fluorotryptophan (Coy et al., 1974) for the synthesis. All peptides were monitored and checked for final purity by reversed-phase high pressure liquid chromatography on a column (0.4 × 25 cm) of C_{18} μ-Bondapack. Good separation of the diastereomers was obtained (220 and 280 nm) by elution with mixtures of the following systems: 10% acetonitrile in 0.01 M NH_4OAc, pH 4.1 (A); and 90% acetonitrile in the same NH_4OAc buffer (B). Conditions were: (1) linear gradient from a mixture of 90% A, 10% B (containing 1% *N*-ethylmorpholine) to 60% A, 40% B over 20 min; (2) linear gradient from a mixture of 90% A, 10% B to 50% A, 50% B over 10 min; and (3) continuous mixture of 40% A, 60% B. The flow rate was 1.5 mL/min.

Enzymatic Hydrolyses. Each analogue (150 μg) was incubated for 16 h at 37 °C with aminopeptidase M (15 μg) in 0.2 N *N*-ethylmorpholine acetate, pH 8.1 (100 μL); Light, 1972; Huang & Tang, 1972). Digests were acidified with one drop of AcOH, frozen, lyophilized, and aliquots were removed for amino acid analysis. For D- and L-5Br-Trp[8]-SS, an aliquot was removed for dansylation prior to acidification.

Dansylation. Products from enzymatic digests were dansylated by the method of Gray (Gray & Hartley, 1963; Gray, 1972) and isolated by two-dimensional chromatography on polyamide thin layers in the two solvent systems described by Woods & Wang (1967). Dansyl amino acids were visualized under an ultraviolet lamp.

GH Bioassays. The analogues were compared with SS for their ability to inhibit the release of radioimmunoassayable GH in vitro from enzymatically dispersed rat anterior pituitary cells as described previously (Vale et al., 1972; Meyers et al., 1977; Gordin et al., 1977).

Results

Peptide Purity and Physical Data. The six somatostatin analogues were isolated in good yield and appeared homogeneous by thin-layer chromatography in four solvent systems (Table I). Optical rotations appear in Table I. Amino acid analyses of acid hydrolysates had the expected ratios for each analogue (Table II). Each analogue was examined by HPLC (220 and 280 nm) and found to produce a single symmetric

TABLE II: Amino Acid Analyses of Acid Hydrolyzed Somatostatin Analogues.[a]

Substituent in position 8	Ala	Gly	½-Cys	Lys	Asp	Phe	X^8	Thr	Ser	NH_3
L-5F-Trp[b]	1.00	1.00	1.80	2.06	0.94	3.08	0.96	1.96	0.88	1.03
L-5F-Trp	1.00	1.02	1.95	1.94	1.02	3.05	1.04	1.96	0.91	1.32
D-5F-Trp	0.98	1.00	1.77	1.91	1.02	2.94	1.01	1.97	0.94	1.42
L-6F-Trp	1.00	1.03	1.98	1.94	1.03	3.05	0.97	1.93	0.87	1.31
D-6F-Trp	1.00	1.02	1.87	1.93	1.05	3.01	1.04	2.00	0.97	1.39
L-5Br-Trp	1.00	1.00	1.92	1.97	1.04	3.00	[c]	1.94	0.89	1.35
D-5Br-Trp	0.99	1.00	1.88	1.91	1.04	2.97	[c]	2.00	0.95	1.34

[a] Samples were hydrolyzed in 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole at 110 °C for 18 h in sealed evacuated tubes. [b] Prepared from Boc-L-5F-Trp to serve as the all-L standard. [c] Not eluted under standard analyzer conditions.

TABLE III: Amino Acid Analyses of Enzymatically Hydrolyzed Somatostatin Analogues.

Substituent in position 8	Ala	Gly	½-Cys	Lys	Asn + Ser	Phe	X^8	Thr	NH_3
L-5F-Trp	1.22	1.00	1.53	1.69	1.42	2.49	0.62	1.87	0.14
D-5F-Trp	1.00	0.54	0.04	0.21	0.14	0.13			0.14
L-6F-Trp	1.06	1.00	1.62	1.73	1.53	2.60	0.89	1.83	0.11
D-6F-Trp	1.07	1.00	0.91	0.57	0.85	0.68			0.13
L-5Br-Trp	1.10	1.00	1.74	1.84	1.76	2.75	*a*	1.89	0.11
D-5Br-Trp	1.00	0.69		0.16	0.18	0.15	*a*		

[a] Not eluted under standard analyzer conditions.

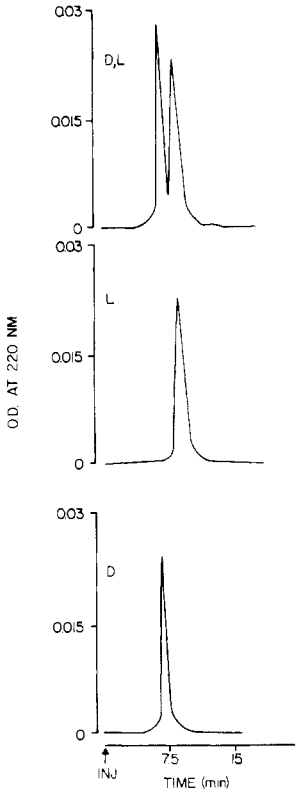

FIGURE 3: HPLC of D- and L-5F-Trp[8]-SS eluted under the isocratic conditions described in the text. Twenty micrograms of each peptide was injected (INJ) in 20 μL of buffer A.

peak under conditions capable of resolving the corresponding diastereomers (e.g., Figure 3).

Identification of Separated Diastereomers. Amino acid analyses of enzymatically digested peptides showed the expected partial cleavage of three analogues (tentatively identified as the D-amino acid containing peptides), and virtually complete cleavage of their corresponding diastereoisomers (Table III). The HPLC elution peaks for D- and L-5F-Trp[8]-SS (Figure 3) were assigned by comparison with authentic L-5F-Trp[8]-SS. The identified D-5F-Trp[8] derivative was partially cleaved by the aminopeptidase as expected and the L diastereomer was totally hydrolyzed (Table III). Thus, the partially digested 5Br- and 6F-Trp[8] analogues could be assigned as their respective D diastereomers, and the totally digested derivatives as their respective L diastereomers. Dansylation and chromatographic separation of enzymatically digested D-5Br-Trp[8]-SS confirmed the presence of asparagine rather than serine, since these amino acids are not separated during amino acid analysis in sodium citrate buffers (Light, 1972; Table III). Dansylation of digested L-5Br-Trp[8]-SS allowed identification of all the expected amino acids, including Asn and Ser, as well as a new spot corresponding to DNS-5Br-Trp traveling below and slightly to the left of DNS-Lys (not shown). 5Br-Trp was not eluted from the amino acid analyzer by the modified single column methodology employed (Coy et al., 1974). 5F- and 6F-Trp were eluted 77 and 102 min after Lys, respectively.

Biological Activity. All six analogues were more active than SS in suppressing pituitary GH release in vitro. The strongest actions were exhibited by D-5F-Trp[8]- and D-5Br-Trp[8]-SS, which had 25 and 30 times greater GH release-inhibiting activity than SS, respectively. The activities of all the analogues appear in Table IV.

Spectrophotometry. Wavelengths of maximum absorption (λ_{max}) for the analogues and SS are reported in Table V.

Discussion

Preparative Methodology. The complete isolation of closely related peptides by conventional purification techniques could have widespread applications. For example, the resolution of racemic radiolabeled amino acids can be difficult and expensive, whereas separation of diastereomeric peptides may pro-

TABLE IV: In Vitro GH Release-Inhibiting Activities of Somatostatin Analogues Relative to Somatostatin (SS).

Substituent in position 8	GH release inhibiting act. (%)
L-Trp (SS)	100
L-6F-Trp	118
D-6F-Trp	846
L-5F-Trp	476
D-5F-Trp	2499
L-5Br-Trp	548
D-5Br-Trp	3002

TABLE V: Absorption Maxima (λ_{max}) of Somatostatin and Analogues.[a]

Substituent in position 8	λ_{max} (nm)
L-Trp (SS)	280
D-Trp[b]	280
L-6F-Trp	281
D-6F-Trp	281
L-5F-Trp	285
D-5F-Trp	285
L-5Br-Trp	289
D-5Br-Trp	289

[a] Peptide samples (1.3×10^{-4} M) in 0.1 M AcOH were read from 275 to 295 nm at 1 nm intervals. [b] This analogue was prepared for an earlier study (Coy et al., 1976).

vide a practical alternative (Yamamoto et al., 1977). Often, as is the case with somatostatin analogues, not only the all-L peptides, but also the peptide stereoisomers containing one or more D-amino acids have important biological effects. Thus, in addition to bypassing the extra steps necessary to resolve racemic amino acids, the foregoing procedure represents a simultaneous preparation of two distinct and potentially interesting peptide analogues from a single solid phase synthesis. Since some of the diastereomers were resolved better than others (e.g., 5Br-Trp^8-SS had the best separation in this series) after one pass through a partition column, complete separation from a single partition column run may be expected for some peptide mixtures (Yamamoto et al., 1977), particularly in conjunction with modifications of the partition solvent systems.

Thin-layer chromatography was inadequate in all systems tested to resolve the diasteromeric mixtures (Table I).[3] Although their separation after partition chromatography could be visualized spectrophotometrically (280 and 300 nm), pooling of fractions on that basis could result in some mixing of incompletely resolved regions or, by avoiding that possibility, in significant losses in yield. Both of these problems were circumvented by monitoring small samples of individual column fractions by reversed-phase HPLC. This application of HPLC has not to our knowledge been previously applied to unprotected peptides, although HPLC had been used recently to separate analytical samples of stereoisomeric somatostatin and other peptides (Burgus & Rivier, 1976). In addition to the above advantages of this application, the procedure usually required no more time than thin-layer chromatography.

Enzyme Hydrolyses. Enzymatic hydrolysis with aminopeptidase M permitted identification of D and L diastereomers, since only the all-L peptides were totally cleaved (Table III). It is interesting that of the D diastereomers, only D-6F-Trp^8-SS was extensively cleaved from residues 1-6, while D-5F- and D-5Br-Trp^8-SS were relatively resistant to cleavage after removal of their N-terminal Ala and Gly residues which comprise the linear portion of these peptides (Figure 1). However, the cleavage which did occur in these analogues was also restricted to the first six residues.[4] It is therefore possible that the very high activities of the D-5F- and D-5Br-Trp^8-SS analogues may partly result from their resistance to enzymatic degradation. These peptides are currently being tested in our laboratory for prolonged activity in vivo.

It is also of interest that aminopeptidase M cleaved the cyclic all-L peptides completely, liberating cysteine rather than cystine as judged by amino acid analysis. That cleavage of the disulfide bond occurred is further evidenced by the appearance of a single cysteine in the enzyme hydrolysate of D-6F-Trp^8-SS (Table III).

Biological Activity. The GH release-inhibiting activities of D-5F-Trp^8- and D-5Br-Trp^8-SS (25 and 30 times that of SS, respectively) are the highest in the literature for somatostatin analogs, despite the fact that each has been modified at only one position. D-Trp^8-SS is only five to eight times as active as SS in suppressing GH release (Rivier et al., 1975; Coy et al., 1976), while D-Ala^2, D-Trp^8-SS is up to 20 times as active as SS (Schally et al., 1977, 1978). By analogy to these and other superactive SS analogues containing D-Trp in position 8 (Meyers et al., 1977) and having observed that multiple substitutions in the structure of SS have produced additive effects on the inhibition of GH release, we may reasonably expect the development of modified Trp^8-containing analogues with still greater activities. Extremely active derivatives, particularly in more selective and long acting forms, could offer clinical advantages in treating certain diseases; e.g., required doses for injection could be sufficiently low to reduce the side effects which occur at higher doses, and the biological activities could be sufficiently high to permit oral or even nasal administration.

Role of Tryptophan in Somatostatin. The activities of the halogenated Trp^8 analogues provide some insight into the importance of Trp in the action of SS on the pituitary. The steric effect of replacing a hydrogen atom with a fluorine atom of similar atomic radius in position 5 or 6 of the aromatic nucleus of Trp is probably minimal. Nevertheless, these analogues exhibited high GH release-inhibiting activities.

The primary importance of the electron-rich tryptophanyl residue in other biologically active molecules, such as in LH-RH peptides (Coy et al., 1974), has been attributed to its role as an electron donor. Thus, in some biologically active compounds, the modification of Trp residues with substituents that tend to deactivate the aromatic indole nucleus by reducing its electron density results in a reduction in biological activity. For example, 5F-Trp^3-LH-RH has predictably low biological activity since fluorine has a net electron-withdrawing effect (Coy et al., 1974). However, the halogenated Trp^8-SS analogues are all more active than SS in inhibiting GH release, suggesting that the electron-withdrawing effects of the halogens are of secondary importance to tryptophan's role in maintaining the biological activity of somatostatin peptides, at least on the pituitary.

[3] A slight separation was consistently observed for all three diastereomeric mixtures in solvent IV (Table I) when applied to silica plates in concentrated solutions.

[4] Asn and Ser elute together during amino acid analysis in sodium citrate buffers (Light, 1972). Proof that Asn^5, and not Ser^{13}, was hydrolyzed by the aminopeptidase was obtained in the dansylation experiment on D-5Br-Trp^8-SS. See text.

The large differences in GH release-inhibiting activity between the 5F-Trp[8]- and the 6F-Trp[8]-SS analogues (D-5F-Trp[8]- and L-5F-Trp[8]-SS are about three and four times stronger than D-6F-Trp[8]- and L-6F-Trp[8]-SS, respectively) could perhaps be explained by alterations in the resonance structures formed when the fluorine atom is at different positions in the aromatic ring. We therefore measured the λ_{max} of each analogue and SS since the most fully conjugated systems should be shifted to longer wavelengths. Table V shows that SS has the least conjugated system (λ_{max} at 280 nm), followed by the 6F-Trp[8]-SS analogues (281 nm), the 5F-Trp[8]-SS analogues (285 nm), and finally the 5Br-Trp[8]-SS analogues (289 nm). The λ_{max} was not affected by replacing the amino acid residues in position 8 with their respective stereoisomers. Although the shifts are modest, there is in each case a direct correlation between the increases in λ_{max} and the observed biological activity of the SS analogues; as the aromatic indole nucleus becomes more fully conjugated, the biological potency increases in both the L and D analogue series.

While viewed here as a primary determinant of biological activity, the degree of resonance in the modified tryptophanyl residues must also affect the relative electron-withdrawing strengths of the halogen atoms. For example, the resonance effect, which opposes the negative inductive effect of the fluorine atom, is stronger (based on the spectral shifts) in 5F-Trp[8]-SS than in 6F-Trp[8]-SS (Table V), so that fluorine in 6F-Trp[8]-SS has a stronger net electron-withdrawing effect than in 5F-Trp[8]-SS. This relative deactivation of the aromatic indole ring system, in terms of its electron donating properties, could account for the lower biological activities of the 6F-Trp[8]-SS analogues compared with the respective 5F-Trp[8]-SS analogues.

There is no electron-withdrawing substituent in the unsubstituted Trp residue of somatostatin; yet SS is less active than all of the halogenated Trp[8]-SS analogues. Therefore, it seems that the positive resonance effects of the halogens more strongly influence the interaction of the indole ring system with the pituitary receptor than do their negative inductive effects. Although the mechanism(s) by which the resonance structures may regulate biological activity remain unclear, it is possible to imagine that a broader area of resonance may facilitate increased overlapping of the electron orbitals with receptor sites. The shifts in λ_{max} to longer wavelengths in the most potent analogues attest to the lower energies required to "activate" the electrons in the aromatic indole ring, which in turn may indicate the existence of loose, more accessible electrons.

Since a steric effect could not account for the differences between 5F- and 6F-Trp[8]-SS derivatives, it probably cannot completely explain the extremely high activities of the 5Br-Trp[8]-SS analogues, even though bromine has a larger radius than fluorine. The 5Br-Trp[8]-SS peptides exhibited the greatest spectral shift and the greatest biological activity. In addition, bromine would be expected to have a much weaker negative inductivity than fluorine, especially when the competing effect of resonance in the ring is considered. These properties are consistent with the above concept that the importance of Trp in the action of somatostatin peptides on the pituitary depends in part upon its strongly conjugated aromatic ring system.

Conformational shifts and possible resultant differences in lipophilicity are difficult to assess and may contribute to the observed biological effects. Indeed, the differing rates of enzymatic hydrolysis between the D diastereomers of 5F-Trp[8]-SS and 6F-Trp[8]-SS underscore the importance of conformational changes. As already mentioned, resistance to enzymatic degradation may also contribute to the high activities of some of the analogues. Nevertheless, even the all-L peptides, which were readily digested by the aminopeptidase, were more active than somatostatin. Thus, besides resistance to enzymatic degradation, additional mechanisms appear to be operative in enhancing the biological activity of the halogenated analogues.

It may be that the halogens serve mainly to promote a more active conformation of SS peptides, as is apparently the case with D-Trp[8]-SS, which does not owe its enhanced actions to substituents in its indole ring or to increased resistance to enzymatic degradation (Coy et al., 1976). It is interesting, however, that each of the D diastereomers is about six times as active as its corresponding all-L analogue (Table IV). D-Trp[8]-SS is also about six times more active than SS (Rivier et al., 1975). These results suggest that in each case the D-amino acid residue in position 8 favorably alters the conformation of the SS peptide in a similar fashion.

Studies with modified Trp[8]-SS analogues are continuing in our laboratory to further test the correlations observed in these experiments, and to detect possible differences between SS receptors in the pituitary and other cell types.

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Human Skin Fibroblast Collagenase: Chemical Properties of Precursor and Active Forms†

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ABSTRACT: Human skin fibroblast procollagenase and the trypsin-activated enzyme species were isolated in pure form and subjected to chemical analysis. Ultracentrifugation confirmed the molecular weight of the zymogens to be 55 000–60 000 and of the active enzyme forms to be 45 000–50 000. Molecular weights determined by sodium dodecyl sulfate gel electrophoresis were in close agreement with these values. Molecular weights estimated from gel filtration on Sephadex G-100 or Ultragel AcA-44 are, therefore, apparently spurious for reasons not presently known. Amino acid analysis of each of the two zymogens revealed essentially no significant compositional differences between the two molecules; only the values for histidine appeared to vary significantly. Similarly, the two tryptically activated molecules appeared to be nearly identical in amino acid composition. Clear differences existed, however, between the composition of zymogen and activated molecules. Trypsin activation of mixed proenzyme species resulted in the loss of a peptide of approximately 10 000 daltons in mass and markedly altered the binding characteristics of these molecules to fibrillar collagen substrates. The procollagenases failed to bind to collagen, whereas the trypsin-activated enzymes bound tightly to the substrate. Cyanogen bromide cleavage of isolated procollagenase indicated that minor differences, possibly involving only one peptide, exist between the two molecular forms. Differences between proenzyme and activated enzyme species were greater, although still limited to a small number of peptides. The similar nature of all the forms of the human skin fibroblast collagenase is further emphasized by the fact that antibody to pure proenzyme reacts with identity to pure activated enzyme species as well.

Recent studies from this laboratory have demonstrated that human skin fibroblasts produce collagenase as a proenzyme and methods have been developed which have allowed the purification of the procollagenase to homogeneity (Stricklin et al., 1977). The resultant pure proteins were shown to comprise a set of two zymogens, each of which could be converted proteolytically to a corresponding active form. Furthermore, an autoactivation process occurred in which, by incubation at 37 °C or by freeze–thawing, each zymogen yielded an active enzyme without a detectable change in molecular weight. Serum-free explant cultures of human skin were shown to produce only enzyme species identical to the trypsin-activated forms, whereas if the explants were grown in the presence of serum only the zymogen forms of collagenase were present in the culture medium.

The proteins of this collagenolytic system behaved anomalously on gel filtration and, depending upon the matrix, a variety of apparent molecular weights from 25 000 to 42 000 could be obtained. These enzymes also displayed unusual electrophoretic properties. Neither the collagenase zymogens nor the active enzyme forms migrated in the standard basic polyacrylamide electrophoresis system of Davis (1974). Although the pure collagenases would migrate in an acidic polyacrylamide electrophoretic system (Reisfield et al., 1962), sharp bands were obtained only by the addition of 8 M urea. Electrophoresis of the zymogen and active enzymes could be obtained on sodium dodecyl sulfate–polyacrylamide gels employing a continuous buffer system (Fairbanks et al., 1971) but gave rise to still a different set of molecular weights. Two forms of the procollagenase were detected whose apparent molecular weights were 60 000 and 55 000. Each form could be converted to active enzyme by trypsin, producing species of 50 000 and 45 000, respectively.

An interesting characteristic of all of the fibroblast collagenase species has been their ability to bind to both anion- and cation-exchange resins under similar conditions. The purification employed (Stricklin et al., 1977) takes advantage of the fact that these proteins bind well to the cation-exchange resins, phosphocellulose, and CM[1]-cellulose, at neutral pH. Woolley and co-workers (1973) have shown that human skin collagenase also binds to QAE-Sephadex at similar pH and salt concentrations.

In order to ascertain the reasons for the differences between the two zymogen forms as well as the unusual behavior of the various enzyme species identified, it is essential to define some of the physical and chemical characteristics of the fibroblast collagenase system. This report presents the results of initial

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[1] Abbreviations used are: CM, carboxymethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl.

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