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Partial Retro–Inverso Analogues of Somatostatin: Pairwise Modifications at Residues 7 and 8 and at Residues 8 and 9†

P. V. Pallai, R. S. Struthers, and M. Goodman*
Department of Chemistry, University of California at San Diego, La Jolla, California 92093
L. Moroder and E. Wunsch
Department of Peptide Chemistry, Max Planck Institute of Biochemistry, 8033 Martinsried, Federal Republic of Germany
W. Vale
Peptide Biology Laboratory, The Salk Institute, San Diego, California 92138
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ABSTRACT: Peptide bonds between residues 7 and 8 and residues 8 and 9, postulated internal cleavage sites of the peptide hormone somatostatin, were subjected to "pairwise" retro–inverso modification, where atoms of these peptide bonds were interchanged to give the analogues [*g*Phe^7-*m*-(*RS*)-Trp^8]somatostatin (I) and [*g*Trp^8-*m*-(*RS*)-Lys^9]somatostatin (II). Key fragments containing the modifications were synthesized by using [bis(trifluoroacetoxy)iodo]benzene for the generation of *gem*-diaminoalkyl-containing precursors from peptide amides. The versatility of solution synthetic methods was utilized to allow the incorporation of the modified segments. Protecting groups, removable selectively and under mild conditions, included *tert*-butyl-based groups for the side chains and the *tert*-butylmercapto group for the cysteine thiols. The excellent results obtained in the syntheses of analogues I and II, and previously of somatostatin on a larger scale [Moroder, L., Gemeiner, M., Goehring, W., Faeger, E., Thamm, P., & Wunsch, E. (1981) *Biopolymers 20*, 17–31], suggest the general feasibility of this route for the synthesis of centrally modified analogues. The purification of the products by Sephadex LH-20 chromatography afforded the separation of diastereomers of both analogues. The two isomers of I showed significant but different activities while those of analogue II were marginally active.

The disulfide-linked cyclic tetradecapeptide somatostatin is an important regulatory factor responsible for the inhibition of the release of growth hormone, insulin, glucagon, gastrin, and other peptide hormones. The in vivo half-life of somatostatin is only a few minutes due to rapid degradation by exo- and endopeptidases (Brazeao et al., 1974). In vitro experiments with brain homogenates suggested that the major internal cleavage site is between residues Trp^8 and Lys^9, in addition to the minor sites between residues Phe^6 and Phe^7 and residues Thr^{10} and Phe^{11} (Marks & Stern, 1975; Marks et al., 1976). These cleavages all occur within the central 6–11 region which has been established through structure activity studies as essential for biological function (Vale et al., 1978)

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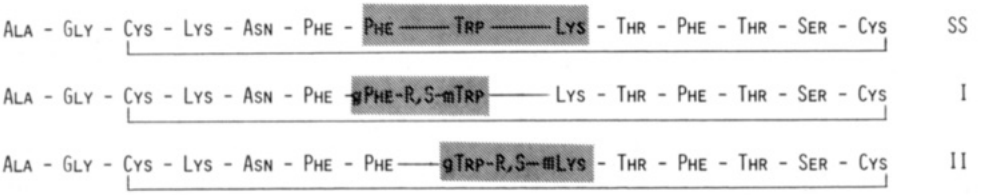
Chart I



and therefore should generate inactive fragments. To prevent cleavage, and thus inactivation, modifications have been sought that result in enzymatic resistance so that the structural–topological features required for eliciting biological response are maintained. Introduction of D-Trp at position 8 (Rivier et al., 1975) and the design of highly constrained cyclic analogues (Veber et al., 1981) have resulted in active analogues with improved resistance to enzymatic degradation.

Partial retro–inverso modification (Goodman & Chorev, 1979) has been applied to enhance enzymatic resistance of a number of peptide hormones and neurotransmitters including enkephalins (Chorev et al., 1979), LHRH (Chaturvedi et al., 1981), substance P (Rubini et al., 1981), and somatostatin (Pallai et al., 1983a) analogues. The reversal of peptide bonds within the entire central portion of somatostatin by application of extended retro–inverso modification resulted in marginal biological activity (Pallai et al., 1983a). The focus of this work is to synthesize two pairwise modified analogues of somatostatin where only the internal scissile peptide bonds between residues Phe[7] and Trp[8] and residues Trp[8] and Lys[9] respectively, are reversed and to assess whether modifications within this conformationally sensitive region are compatible with biological activity. Reversal of the peptide bond between residues 7 and 8 of somatostatin gave [*g*Phe-*m*-(*RS*)-Trp]somatostatin (I)[1] and between residues 8 and 9 gave [*g*Trp-*m*-(*RS*)-Lys]-somatostatin (II) (Chart I).

Experimental Procedures

Melting points were measured with a Thomas–Hoover capillary melting point apparatus and are uncorrected. The optical rotations were measured on a Perkin-Elmer Model 141 digital readout polarimeter. HPLC analyses were performed by a Waters gradient HPLC system equipped with a Model 660 solvent programmer, two M45 solvent pumps, a Kratos SF 74 UV detector operating at 215 nm, and a Hewlett-Packard 3390A reporting integrator. Unimetrix reverse-phase (Lichosorb RP-18, 7 μm) 4 mm × 25 cm columns were employed for analytical samples. Semipreparative separations were carried out on Whatman (Partisil M9 ODS-3) 9.4 mm × 25 cm columns. The following were the compositions of the HPLC solvent systems: (i) buffer A, 0.25 N triethylammonium phosphate, pH 2.25; buffer B, 40% A and 60% acetonitrile (Rivier, 1978); (ii) buffer A, 0.1% trifluoroacetic acid; buffer B, 40% buffer A and 60% acetonitrile.

Thin-layer chromatography was performed on silica gel 60 and HPTLC plates (Merck AG, Darmstadt, Germany) using the following systems: A, $CHCl_3$–MeOH (9:1); B, EtOAc–hexanes (1:1); C, $CHCl_3$–MeOH–AcOH (85:10:5); D, $CHCl_3$–MeOH–AcOH (9:1:0.02); E, 1-BuOH-AcOH-H_2O (4:1:1); F, 1-BuOH–AcOH–EtOAc–H_2O (1:1:1:1); G, $CHCl_3$–MeOH (4:1); H, $CHCl_3$; I, $CHCl_3$–acetone–AcOH (9:1:1); J, $CHCl_3$–MeOH–concentrated ammonia (aqueous) (18:9:2); K, $CHCl_3$–EtOAc–AcOH (9:1:0.02); L, hexane–1-BuOH–AcOH (3:2:1); M, 1-BuOH–AcOH–pyridine–H_2O (30:10:15:20). A standard aqueous workup consists of extractions of the material dissolved in an appropriate organic solvent with $NaHSO_4$ solution, water, brine, 10% aqueous $NaHCO_3$, and brine.

All materials were of reagent grade and used without further purification with the following exceptions: HOBt was recrystallized from MeOH, and HOSu was recrystalized from EtOAc; [bis(trifluoroacetoxy)iodo]benzene was freshly synthesized (Maletina et al., 1974); DCC, TFA, DMF, and THF were distilled.

The synthesis of analogues I and II are outlined in Figures 1 and 3, respectively. The synthesis of compounds I-1 and I-5 and compounds II-2, II-7, II-8, II-9, II-11, and II-12 are described in the supplementary material (see paragraph at end of paper regarding supplementary material).

Synthesis of Analogue I[2]

Boc-Phe-gPhe-H·TFA (I-2). Dipeptide amide I-1 (4.94 g, 12 mmol) was treated with [bis(trifluoroacetoxy)iodo]benzene (5.42 g, 12.36 mmol) in a mixture of acetonitrile (90 mL) and water (30 mL). After 3 h of intensive stirring the acetonitrile was evaporated under reduced pressure, and the aqueous solution was frozen and lyophilized. The crude dry material was triturated with isopropyl ether and filtered: yield 5.49 g (92%); mp 132–135 °C; $[\alpha]^{25}_D$ −42.0° (*c* 1, MeOH); R_f 0.52 (C), 0.37 (I). Anal. Calcd for $C_{24}H_{30}N_3O_5F_3$ (M_r 497.51): C, 57.94; H, 6.08; N, 8.44. Found: C, 57.90; H, 6.04; N, 8.31.

HO-(RS)-mTrp-OEt (I-3). Gramine (26.1 g, 150 mmol), diethyl malonate (68.25 mL, 450 mmol), and NaOH (1.8 g) were dissolved in xylenes (180 mL) in a 1-l round-bottom flask equipped with a water-cooled condenser. The mixture was refluxed under a N_2 atmosphere with stirring for 4 h. The reaction was acidified with concentrated HCl, and an aqueous workup was carried out to give an oil. The product was stirred under hexanes and the supernatant decanted; repeating this procedure several times gave a filterable solid: yield 33.4 g (77%); mp 53–56 °C; R_f 0.49 (B).

The diester (7.225 g, 25 mmol) was dissolved in EtOH containing KOH (25 mmol), and the reaction was stirred under a N_2 atmosphere for approximately 6 h. The ethanol was removed under reduced pressure, and the aqueous remainder was distributed between aqueous $NaHCO_3$ and ether/isopropyl ether. The aqueous layer was collected and the organic washed again with $NaHCO_3$. Aqueous layers were pooled and washed with ether/isopropyl ether and then acidified and extracted several times with ether. Ether layers

[1] Abbreviations: Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; Bu^t, *tert*-butyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; DCU, *N,N'*-dicyclohexylurea; DMF, *N,N*-dimethylformamide; EDT, ethanedithiol; EtOAc, ethyl acetate; For, formyl; *g*Phe, 1,1-diamino-2-phenylethyl; *g*Trp, 1,1-diamino-2-(3-indolyl)ethyl; HOBt, 1-hydroxybenzotriazole; HOSu, hydroxysuccinimide; HPTLC, high-performance thin-layer chromatography; HPLC, high-performance liquid chromatography; IPE, diisopropyl ether; *m*Trp, (3-indolyl)methylmalonyl; TBP, tributylphosphine; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; THF, tetrahydrofuran; TIB, [bis(trifluoroacetoxy)iodo]benzene; Z, benzyloxycarbonyl.

[2] Syntheses of fragments 1–3 and 12–14 are described elsewhere (Moroder et al., 1981).

were pooled, washed with acid and then brine, and dried over $MgSO_4$. After reduction of the solvent, the addition of DCHA (5 mL) and trituration under hexanes gave the solid salt: yield 1.02 g (87%); R_f 0.40 (G). Dissolving in EtOAc/ether, washing with aqueous $NaHSO_4$, and removing the solvents gave an oil that solidified on standing: mp 93–96 °C; R_f 0.53 (C), 0.76 (D). Anal. Calcd for $C_{14}H_{15}O_4N_1$ (M_r 261.28): C, 64.36; H, 5.79; N, 5.36. Found: C, 64.24; H, 5.86; N, 5.41.

Boc-Phe-gPhe-(RS)-mTrp-OEt (I-4). The free monoester of malonyltryptophan (2.19 g, 8.4 mmol) and dipeptide I-2 (2.98 g, 6 mmol) were dissolved in DMF. *N*-Methylmorpholine (0.672 mL) was added and the solution cooled to 0 °C before HOSu (980 mg) and DCC (1.76 g) were added. The reaction mixture was stirred for 30 min and then at room temperature overnight, when 10% more DCC was added. On completion of the coupling, the DMF was removed under reduced pressure and the urea filtered from EtOAc. After a standard aqueous workup and removal of the solvents, the trituration under isopropyl ether gave a solid material: yield 3.23 g (85%); mp 158–163 °C; $[\alpha]^{25}_D$ 3.1° (*c* 1, DMF); R_f 0.85 (C). Anal. Calcd for $C_{36}H_{42}N_4O_6 \cdot 0.75H_2O$ (M_r 640.26): C, 67.53; H, 6.85; N, 8.75. Found: C, 67.49; H, 6.93; N, 8.99.

Z-Asn-Phe-gPhe-(RS)-mTrp-OH (I-6). Deprotected tripeptide I-5 (361 mg, 0.58 mmol) was dissolved in DMF, and Z-Asn-ONp (236 mg, 0.61 mmol) was added. The reaction mixture was cooled to 0 °C, and triethylamine (161 μL) and pyridine (47 μL) were added. Stirring was continued overnight at room temperature. The product was then precipitated with dilute $KHSO_4$ and filtered. This material was redissolved in DMF and precipitated again with water: yield 395 mg (90%); mp 217–221 °C; $[\alpha]^{20}_D$ −12.9° (*c* 1, DMF); R_f 0.69 (C). Anal. Calcd for $C_{41}H_{42}N_6O_8 \cdot 2H_2O$ (M_r 782.85): C, 62.92; H, 5.92; N, 10.74. Found: C, 61.69; H, 5.92; N, 11.44.

Nps-Lys(Boc)-Asn-Phe-gPhe-(RS)-mTrp-OH (I-7). Benzyloxycarbonyl tetrapeptide I-6 (1 g, 1.32 mmol) was dissolved in DMF (21 mL), MeOH (42 mL), and H_2O (2.1 mL) and stirred intensively with palladium black in a slightly pressurized H_2 atmosphere. Disappearance of the starting material was followed by TLC (solvent C). On completion of the reaction, the solution was filtered through Celite, and the solvents were removed in vacuo. The product was filtered from cold CH_3CN. Washing with ether/isopropyl ether gave a white powder: yield 632 mg (77%); mp 152–165 °C; $[\alpha]^{20}_D$ 3.3° (*c* 1, DMF); R_f 0.37 (E).

The resulting free tetrapeptide analogue (2.515 g, 4.04 mmol) was dissolved in DMF containing triethylamine (561 μL) and pyridine (343 μL) and cooled to 0 °C. Nps-Lys-(Boc)-OSu (2.10 g, 4.24 mmol) was added, and the reaction was stirred for 2 h at 0 °C and then at room temperature overnight. The product precipitated from the reaction mixture on addition of dilute aqueous $NaHSO_4$ and was filtered by washing extensively with water followed by ether and isopropyl ether to give 3.19 g of material. This was dissolved in a minimum amount of DMF and reprecipitated with ether: yield 3.23 g (82%); mp 209–215 °C; $[\alpha]^{20}_D$ −8.2° (*c* 1, DMF); R_f 0.38 (C). Anal. Calcd for $C_{50}H_{59}N_9O_{11}S_1 \cdot H_2O$ (M_r 1085.24): C, 58.66; H, 6.32; N, 12.91. Found: C, 58.58; H, 5.98; N, 12.71.

Nps-Lys(Boc)-Asn-Phe-gPhe-(RS)-mTrp-Lys(Boc)-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-Cys(SBu^t)-OBu^t (I-8). The protected hexapeptide (Moroder et al., 1981), H-Lys(Boc)-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-Cys(SBu^t)-OBu^t (550 mg, 0.52 mmol) and retro–inverso pentapeptide I-7 (520 mg, 0.52 mmol) were dissolved in 10 mL of DMF and cooled to 0 °C. Addition of HOSu (69 mg) and DCC (113 mg) was followed by stirring at 0 °C for 4 h and then at room temperature for 48 h. Insoluble urea was filtered, and the DMF was removed under reduced pressure. The material was filtered from EtOAc, triturated in CH_3CN, and collected by filtration: yield 850 mg (82%); mp 210–213 °C; $[\alpha]^{20}_D$ −8.6° (*c* 1, DMF); R_f 0.78 (A). Anal. Calcd for $C_{104}H_{152}N_{16}O_{22}S_3$ (M_r 2074.61): C, 60.21; H, 7.38; N, 10.80. Found: C, 59.82; H, 7.37; N, 10.76.

Boc-Ala-Gly-Cys(SBu^t)-Lys(Boc)-Asn-Phe-gPhe-(RS)-mTrp-Lys(Boc)-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-Cys(SBu^t)-OBu^t (I-9). Nps-protected peptide *I-8* (700 mg, 0.33 mmol) was dissolved in DMF containing 2-methylindole (870 mg). The solution was cooled to 0 °C, and HCl (2.9 mL, 0.25 N in MeOH) was added in portions of 200 μL every 2 min. The reaction was then allowed to warm to room temperature, and the cleavage was complete within 30 min. The product was precipitated by addition of isopropyl ether/hexane (1:1) and filtered to give 651 mg of product. This was dissolved in a small amount of DMF and reprecipitated with dilute $NaHCO_3$ to give the free amine: yield 618 mg (97%); mp 205–209 °C; $[\alpha]^{25}_D$ −15.0° (*c* 1, DMF); R_f 0.43 (G).

Boc-Ala-Gly-Cys(SBu^t)-OH (284.4 mg, 0.65 mmol) and the free 11-peptide amine (960 mg, 0.5 mmol) were dissolved in 25 mL of DMF. The solution was cooled to −15 °C, and HOSu (75 mg, 0.65 mmol) and DCC (134 mg, 0.65 mmol) were added. The reaction was allowed to proceed at −15 °C for 30 min, 0 °C for 3 h, and room temperature for 2 days. The solvent was partially reduced, and the crude material was precipitated by addition on $NaHSO_4$ solution, collected by filtration, and washed with water followed by isopropyl ether/hexane. The product was triturated in warm EtOH, centrifuged, and filtered from IPE: yield 935 mg (80%); mp 224–226 °C; R_f 0.69 (C), 0.45, and 0.48 (A); Amino acid analysis gave Asp 1.0, Thr 1.94, Ser 0.81, Gly 0.97, Ala 1.03, Phe 1.93, and Lys 1.84.

Deprotection and Cyclization (I-11). Protected tetradecapaptide I-9 (500 mg, 0.214 mmol) was dissolved in 50 mL of trifluoroethanol (previously flushed with argon for 30 min) and treated with 20 equiv (1.06 mL, 4.28 mmol) of tributylphosphine, stirred for 5 min, and allowed to stand overnight. Most of the trifluoroethanol was evaporated under reduced pressure; 150 mL of EtOAc followed by 70 mL of EtOAc–petroleum ether (2:1) were added to precipitate the product, which was filtered, and washed with EtOAc–petroleum ether (2:1) and petroleum ether. The protected dithiol product was dried under high vacuum. The absence of a S-Bu^t groups was established by NMR. This product was added to a TFA–EDT solution (20 mL of TFA and 4 mL of EDT were mixed and kept at 0 °C for 15 min while argon was bubbled through). Deprotection was allowed to take place for 2 h. Addition of diethyl ether followed by petroleum ether precipitated deprotected tetradecapeptide analogue I-10, yield 424 mg.

The deprotected tetradecapaptide crude product (315 mg) was added in small portions into 3 L of pH 6.8 NH_4OAc buffer containing 1 mL of toluene. The cyclization reaction was followed by the Ellman reaction and was considered completed when the measured SH concentration no longer decreased, i.e., after 6 days. The solution was filtered through the glass filter and lyophilized. Repeated lyophilization yielded 280 mg of white fluffy crude material. TLC showed two bands with some streaking: R_f 0.48 and 0.53 (M); HPLC (two major peaks) R_t 20.25 and 22.47 min, solvent system i, 30 → 60B in 30 min.

Purification. The cyclized crude product (190 mg) was dissolved in a total of 40 mL of 0.1 M NH_4OAc, pH 6.0, buffer, and the solution was passed through a Millipore filter (0.22 μm) to remove insoluble material. The filtrate was loaded onto a Sephadex LH-20 (3 × 200 cm) column equilibrated with the same buffer and eluted at a 25 mL/h flow rate. The elution profile as monitored by UV absorption at 254 nm is shown on Figure 2. Fractions corresponding to nine zones were lyophilized and analyzed by TLC and HPLC. By both methods, two major components, IA and IB, were detected, which were in zones 4–6 (46.0 mg) and 9 (24.2 mg) respectively. Amino acid analysis gave (A) Asp 1:01, Thr 1.91, Ser 0.94, Gly 1.01, Ala 1.00, Phe 1.95, and Lys 1.74, and (B) Asp 1.05, Thr 1.89, Ser 0.82, Gly 1.02, Ala 0.98, Phe 1.92, and Lys 1.80: mass spectrum, *m/z* (FAB) IA, MH^+ = 1638; IB, MH^+ = 1638 (M_r 1637).

Portions of crude IA and IB were further purified by semipreparative HPLC in 5-mg portions. Fractions of the main peak were screened by analytical HPLC for purity, and fractions which were >99% pure was pooled. Desalting of these solutions was carried out by using the TFA/CH_3CN system (solvent system ii), and only the top portion of the peak was collected. Lyophilization gave purified products IA and IB; R_f 0.48 and 0.53 (M); HPLC R_t 20.25 (IA) and 22.47 min (IB) solvent system i, 30 → 60B in 30 min.

Synthesis of Analogue II

DCHA·HO-mLys(Boc)-OEt (II-1). The precursor 4-amino-1-butanol (1.856 mL, 20 mmol) was treated with PCl_5 (5.41 g, 26 mmol) in $CHCl_3$ in the presence of $CaCO_3$ (Carman & Shaw, 1976) (1.0 g, 10 mmol) to give the 4-aminochlorobutane hydrochloride which was used without isolation. It was added to a solution of *tert*-butyloxycarbonyl anhydride (4.8 g, 22 mmol) in 20 mL of dioxane and water (20 mL) containing 2.12 g of Na_2CO_3. The reaction mixture was intensively stirred overnight, and Na_2CO_3 was added as necessary to maintain a basic pH. On completion of the reaction, the solution was washed several times with ether. The ether layer was washed with dilute $NaHSO_4$ and brine and dried over $MgSO_4$. The ether was removed under reduced pressure to give a clear oil: yield 3.385 g; R_f 0.58 (H), 0.84 (C).

Condensation with diethyl malonate was carried out by dissolving sodium metal in 15 mL of EtOH (freshly distilled from Mg) followed by the dropwise addition of diethyl malonate (6.072 mL, 40 mmol) and *tert*-butyloxycarbonyl-4-aminochlorobutane. The mixture was refluxed for 8 h, the solvents were concentrated under reduced pressure, and an aqueous workup was carried out. Removal of the ether under reduced pressure gave an oil which contained significant amounts of diethyl malonate and other impurities. The product was purified by flash chromatography using a silica column (5 × 16 cm) eluted with chloroform. The fractions were analyzed on TLC (H). This procedure gave a light clear oil: yield 1.63 g; R_f 0.9 (H), 0.79 (C).

The diester (2.806 g, 8.47 mmol) was dissolved in EtOH, and an equimolar amount of KOH was added. The mixture was stirred intensively for 2 h at room temperature. The EtOH was then removed under reduced pressure and the residue distributed between $NaHCO_3$ (10% aqueous) and isopropyl ether. The aqueous layer was collected, acidified and then extracted with ether several times. Organic layers were pooled, washed with acid and brine, and then dried over $MgSO_4$. The ether was removed under reduced pressure to give an oil. Dicyclohexylamine (1.694 mL) was added to the oil under hexanes. Heating was followed by rapid cooling to −15 °C and trituration. This procedure was repeated several times to give a solid which was filtered from hexanes: yield 3.146 g (77%); mp 88–91 °C; R_f 0.60 (C), 0.52 (A). Anal. Calcd for $C_{26}H_{48}N_2O_6$ (M_r 484.67): C, 64.43; H, 9.98; N, 5.78. Found: C, 63.69; H, 9.63; N, 5.66.

EtO-mLys(Boc)-D-Trp(For)-NH₂ (II-3). DCHA·HO-mLys(Boc)-OEt (2.903 g, 6.04 mmol) was liberated by washing its ether solution with $NaHSO_4$ solution to give the free acid. It was dissolved in DMF/THF, and NH_2-D-Trp-(For)-OH·HCl (II-2) (1.509 g, 5.66 mmol) was added followed by *N*-methylmorpholine (690 μL). The reaction was then cooled to −15 °C, and HOBt (818 mg) and DCC (1.246 g) were added. Stirring continued at −15 °C for ~1 h, then at 0 °C for 3–4 h, and overnight at room temperature; the DMF was removed under reduced pressure, and DCU was filtered from EtOAc. An aqueous workup from EtOAc gave the product which was collected by precipitation from cold ether: yield 2.30 g (78%); mp 109–113 °C; $[\alpha]^{20}_D$ −7.1° (*c* 1, DMF); R_f 0.60 (A. Anal. Calcd for $C_{26}H_{36}N_4O_7$ (M_r 516.59): C, 60.45; H, 7.02; N, 10.85. Found: C, 60.31; H, 6.94; N, 11.01.

TFA·H-gTrp(For)-mLys(Boc)-OEt (II-4). Malonyl dipeptide amide II-3 (2.58 g, 5 mmol) was dissolved in CH_3CN–water (6:4) (15 mL) and treated under a N_2 atmosphere with TIB (2.36 g, 5.5 mmol) for 4 h. CH_3CN was reduced under vacuum, and the frozen solution was lyophilized. Diethyl ether was added to the dry material and decanted after trituation; this procedure was repeated. Upon addition of isopropyl ether the product solidified and was collected by filtration after cooling: yield 2.70 g (90%); R_f 0.24 (D), 0.28 (A). Anal. Calcd for $C_{27}H_{37}N_4O_8F_3 \cdot 0.75H_2O$ (M_r 616.11): C, 52.64; H, 6.30; N, 9.09. Found: C, 52.81; H, 6.13; N, 9.19.

Nps-Phe-gTrp(For)-mLys(Boc)-OEt (II-5). Nps-Phe-OH (1.057 g, 3.32 mmol) was dissolved in THF and cooled to −15 °C. *N*-Methylmorpholine (372 μL) and isobutyl chloroformate (445 μL) were then added, and activation was allowed to proceed for ~2 min. A solution of dipeptide analogue II-4 (2.0 g, 3.32 mmol) in THF and *N*-methylmorpholine (372 μL) was cooled to −15 °C and added to the activated phenylalanine. The reaction was stirred for 15 min at −15 °C and then at 0 °C for 1.5 h. After an additional 30 min at room temperature the reaction was complete. The THF was then removed and the residue dissolved in EtOAc. This was washed at 0 °C with dilute $NaHSO_4$ and brine, then dried over Na_2SO_4, and decanted. The solution was concentrated under reduced pressure and the product filtered from isopropyl ether: yield 2.165 g (83%); mp 132–136 °C $[\alpha]^{20}_D$ 21.5 ° (*c* 1, DMF); R_f 0.69 (A), 0.21 (K), 0.79 (C). Anal. Calcd for $C_{40}H_{48}N_6O_9S_1$ (M_r 788.91): C, 60.90; H, 6.13; N, 10.65; S, 4.06. Found: C, 60.67; H, 6.27; N, 10.65; S, 4.00.

Nps-Phe-gTrp-mLys(Boc)-OH (II-6). The ethyl ester and N^{in}-formyl groups were removed in a single step from tripeptide II-5 (3.21 g, 4.07 mmol) by treatment with KOH (8.14 mmol) in ethanol. The reaction was stirred under nitrogen for ~7 h, and then additional KOH (0.8 mmol) was added. On completion of the reaction the EtOH was removed under reduced pressure, and the residue was dissolved in EtOAc, washed with $NaHSO_4$ and brine, and then dried over $MgSO_4$. Removal of the EtOAc under reduced pressure gave the solid product which was filtered from ether/isopropyl ether and was Erlich positive on TLC: yield 2.53 g (85%); mp 108–115 °C; $[\alpha]^{20}_D$ 24.6° (*c* 1, DMF); R_f 0.27 (I), 0.31 (A). Anal. Calcd for $C_{37}H_{44}N_6O_8S_1$ (M_r 820.95): C, 59.99; H, 6.38; N, 10.24. Found: C, 59.88; H, 6.49; N, 10.55.

Nps-Phe-gTrp-mLys(Boc)-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-Cys(SBu^t)-OBu^t (II-10). Tripeptide II-6 (996 mg, 1.36

mmol) and pentapeptide II-9 (1.175 g, 1.295) were dissolved in DMF containing *N*-methylmorpholine (145 μL). The solution was then cooled to 0 °C, and HOSu (171 mg) and DCC (307 mg) were added; the coupling proceeded overnight, DMF was removed under reduced pressure, and residue was dissolved in EtOAc, the insoluble urea was filtered, and an aqueous workup was performed. The solution was dried over Na_2SO_4 and the volume reduced. The product precipitated on addition of hexanes: yield 1.695 g (83%); mp 125–135 °C; $[\alpha]^{20}_D$ 10.7° (*c* 1, DMF); R_f 0.78 (I), 0.55 (A). Anal. Calcd for $C_{80}H_{17}H_{11}O_{16}S_3 \cdot 2H_2O$ (M_r 1621.08): C, 59.27; H, 7.52; N, 9.50; S, 5.93. Found: C, 59.37; H, 7.22; N, 9.84; S, 6.99.

Nps-Lys(Boc)-Asn-Phe-OH (II-13). Z-Asn-Phe-OH (3.1 g, 7.5 mmol) was dissolved in DMF/MeOH/H_2O and stirred with palladium black under a H_2 atmosphere for 1.5 h. On disappearance of the starting material (monitored by TLC) solvents were removed under reduced pressure. The residue was dissolved in dilute HCl and lyophilized to give an oil. Trituration under EtOAc gave a filterable solid: yield 2.13 g (90%); R_f 0.26 (E), 0.37 (L).

Nps-Lys(Boc)-OSu (3.336 g, 6.72 mmol) and dipeptide II-12 (2.014 g, 6.4 mmol) were dissolved in DMF and stirred with triethylamine (889 μL) and pyridine (543 μL) overnight. The product was precipitated at 0 °C by addition of dilute aqueous $NaHSO_4$. The material was filtered washing extensively with water and isopropyl ether, yield 3.86 g (91%). This material contained an impurity (higher moving and yellow on TLC) which was not the Nps-Lys(Boc)-OH or its succinimide ester. This impurity was removed in a sample of the product by formation of the DCHA salt and crystallization from DMF/EtOAc: mp 175–182 °C; $[\alpha]^{20}_D$ 4.5° (*c* 1, DMF); R_f 0.49 (J), 0.27 (C). Anal. Calcd for $C_{30}H_{40}N_6O_9S_1 \cdot H_2O$ (M_r 678.76): C, 53.09; H, 6.24; N, 12.38; S, 4.72. Found: C, 52.15; H, 5.97; N, 12.02; S, 5.01.

Nps-Lys(Boc)-Asn-Phe-Phe-gTrp-mLys(Boc)-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-Cys(SBu^t)-OBu^t (II-14). Tripeptide II-13 (509 mg, 0.77 mmol) and octapeptide amine II-11 (1.081 g, 0.755 mmol) were dissolved in DMF and cooled to −15 °C. HOSu (91 mg) and DCC (164 mg) were then added, and the reaction was stirred under nitrogen for 20 min at −15 °C, 2 h at 0 °C, and 5 h at room temperature. DMF was then reduced and the product precipitated on addition of dilute $NaHCO_3$. The solid was filtered, washing with water followed by isopropyl ether and hexanes: yield 1.275 g (82%); mp 175–186 °C; $[\alpha]^{25}_D$ −4.5° (*c* 1, DMF); R_f 0.81 (C), 0.69 (A). Anal. Calcd for $C_{104}H_{152}N_{16}O_{22}S_3$: C, 60.21; H, 7.38; N, 10.80; S, 4.64. Found: C, 59.94; H, 7.90; N, 10.25; S, 5.15.

Boc-Ala-Gly-Cys(SBu^t)-Lys(Boc)-Asn-Phe-Phe-gTrp-mLys(Boc)-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-Cys(SBu^t)-OBu^t·HCl (II-15). Nps-protected peptide II-14 (1.215 g, 0.586 mmol) was dissolved in DMF (13.5 mL) containing 2-methylindole (1.53g) and cooled to 0 °C. HCl (0.25 N) in MeOH (2.46 mL, 0.615 mmol) was added in small aliquots over the course of ~30 min. Stirring continued under nitrogen, and the reaction was allowed to warm to room temperature. When no starting material remained, the DMF was removed under reduced pressure and the product filtered from ether/isopropyl ether: yield 1.02 g (87%); R_f 0.40 (G).

The undecapeptide amine hydrochloride (600 mg, 0.306 mmol) was dissolved in DMF, and *N*-methylmorpholine (34.4 μL, 0.306 mmol) and 1.25 equiv of the protected N-terminal tripeptide (167.4 mg, 0.38 mmol) were added. The solution was cooled to −15 °C, treated with HOSu (4.6 mg, 0.4 mmol) and DCC (82.00 mg, 0.4 mmol), and stirred overnight at −15 °C and subsequently 8 h at room temperature. Upon addition of EtOAc and cooling, the product precipitated and was filtered and washed with water. The crude material was triturated in hot EtOH, cooled, and collected by centrifugation. This procedure was repeated to give the purified, fully protected tetradecapeptide II-15: yield 637 mg (89%); R_f double bands 0.49 and 0.53 (A) nanoplates. Amino acid analysis gave Asp 1.11, Thr 2.23, Ser 0.99, Gly 1.00, Ala 1.00, Phe 2.98, and Lys 0.97.

Deprotection and Cyclization (II-16). The *tert*-butylmercapto groups of the cysteines were cleaved from the protected tetradecapeptide II-15 (350 mg, 0.15 mmol) in TFE with 20 equiv of TBP. The removal of the other *tert*-butyl groups were carried out in the same manner as described for compound I-10 of analogue I.

Deprotected peptide II-16 (256 mg), crude product, was added in small portions over a course of 45 min to 3 L of NH_4OAc buffer (0.1 M, pH 6.8) and was allowed to cyclize for 6 days. The solution was filtered and lyophilized to yield 212 mg of crude product.

Purification. The cyclized crude material was added into 30 mL of NH_4OAc buffer (0.1 M, pH 6.0). The insoluble portion was removed by Millipore filtration (0.22 μm), and the filtrate was applied onto a Sephadex LH-20 column (3 × 197.5 cm) and eluted with the same buffer, as shown in Figure 4. HPLC and TLC analysis of the lyophilized materials corresponding to zones 1–8 identified zone 5 (fractions 76–81, 1014–1090 mL) and zone 7 (fractions 88–92, 1165–1228 mL) as the two separated diastereomeric products IIA and IIB, respectively: yield (IIA) 30.3 mg, (IIB) 14.9 mg; R_f (IIA) 0.58, (IIB) 0.62 (M); Amino acid analysis gave for IIA (fractions 76–81) Asp 1.08, Thr 1.99, Ser 0.90, Gly 1.01, Ala 1.00, Phe 2.92, and Lys 1.00 and for IIB (fractions 88–92) Asp 1.08, Thr 1.95, Ser 0.88, Gly 1.01, Ala 0.99, Phe 2.91, and Lys 1.00: mass spectrum (FAB) (IIA) MH^+ = 1638, (IIB) MH^+ = 1638 (M_r 1637).

IIA was 95% pure by HPLC; it was purified by semipreparative HPLC in two steps: in the first solvent system (i) and a gradient of 35 → 50B in 22 min werre used, and in the second, desalting was carried out by using solvent system ii and a gradient of 35 → 45B in 20 min to give IIA in >98% purity. Similarly, IIB, 93% pure after Sephadex chromatography, was further purified by HPLC in two steps: solvent system i, gradient 35 → 45B in 25 min and desalting by solvent system ii. Repeated lyophilization gave the final product which was 98% pure by HPLC.

Results and Discussion

In the syntheses of the partial retro–inverso analogues I and II key components containing the retro–inverso segments were synthesized and subsequently incorporated into larger fragments. This stragegy allowed us to deal with the unique chemistry involved in the generation *gem*-diaminoalkyl derivatives and incorporation of 2-alkylmalonyl components at the di- and tripeptide level. Conventional peptide synthetic methods could then be applied at more advanced stages of the synthesis. Side chains were protected with *tert*-butyl based groups, and the *tert*-butylmercapto group was employed for the cysteine thiols (Moroder et al., 1981). Benzyloxycarbonyl, nitrophenylsulfenyl, or *tert*-butyloxycarbonyl groups served as complementary α-amino protection.

The synthesis of analogue I is outlined in Figure 1. The precursor of the fragment incorporating the retro–inverso segment was dipeptide amide I-1, which was converted to the *gem*-diaminoalkyl component Boc-Phe-*g*Phe (I-2) by [bis-(trifluoroacetoxy)iodo]benzene (TIB) (Pallai & Goodman, 1982). The classical gramine route was chosen for the prep-

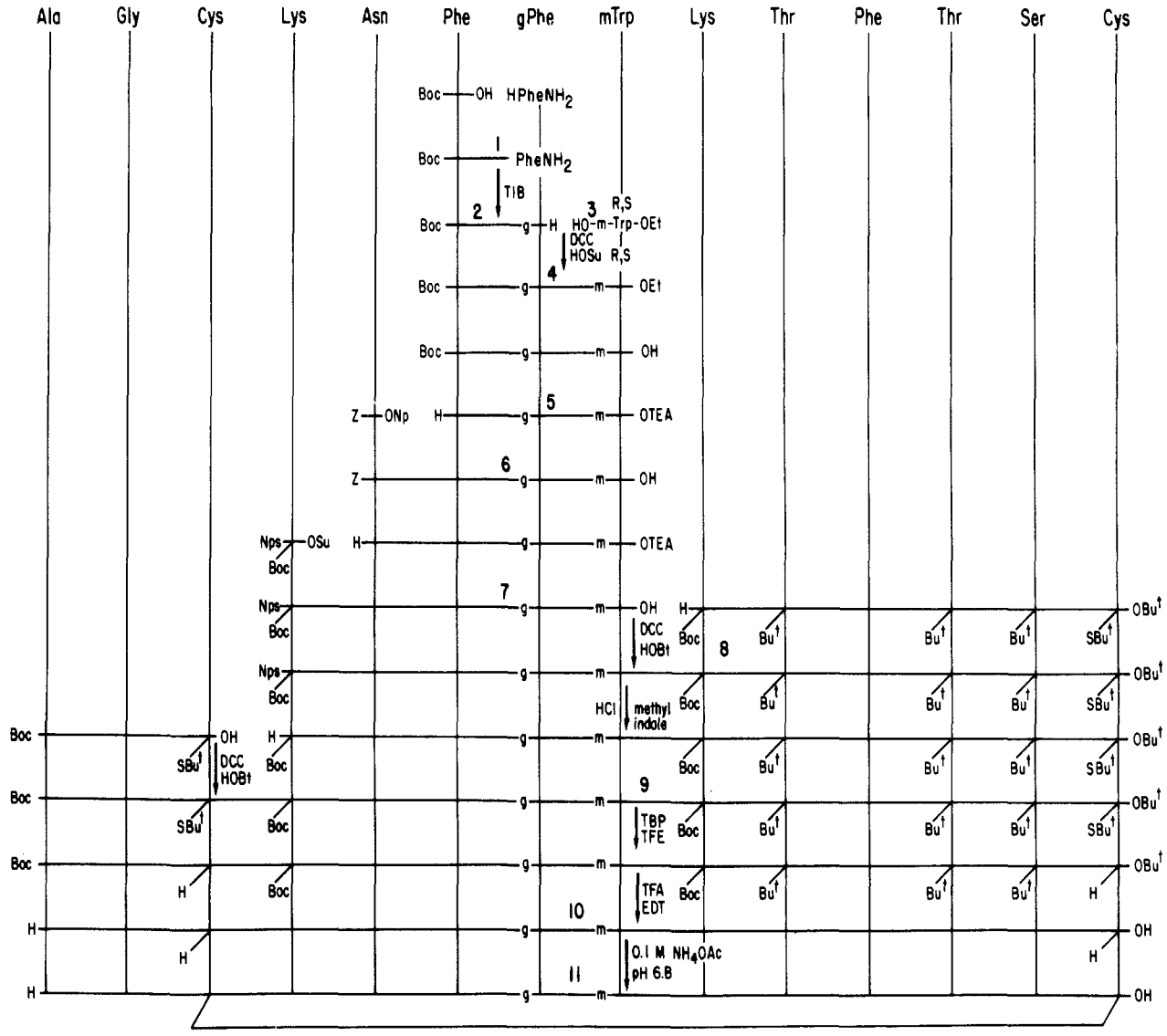


FIGURE 1: Scheme for the synthesis of analogue I.

aration of the diester of *m*Trp (Howe et al., 1945). The ethyl ester of *m*Trp was saponified at the tripeptide level to avoid the sluggish hydrolysis observed for malonyl peptides of larger size. In the two subsequent steps, "salt coupling" with the active ester derivatives of asparagine and lysine was the method of choice. Dicyclohexylcarbodiimide/hydroxysuccinimide-mediated fragment coupling to the C-terminal hexapeptide amine [synthesized previously (Moroder et al., 1981)] was followed by removal of the Nps group by treatment with HCl in the presence of 2-methylindole. The undecapeptide amine was subsequently coupled with the N-terminal tripeptide to give the fully protected tetradecapeptide.

As a result of optimization of deprotection steps in the previous syntheses of somatostatin (Moroder et al., 1981), we were able to utilize this highly effective scheme in which first the *tert*-butylmercapto groups on cysteines are removed by tributylphosphine in trifluoroethanol followed by the acidolytic cleavage of all other protecting groups. This method afforded the deprotected species I-10 in high yield and purity. Cyclization of the dihydro intermediate was allowed to occur for 6 days (pH 6.8, 0.1 M NH_4OAc), and the crude material was subjected to Sephadex LH-20 chromatography (Figure 2). Fractions were pooled according to the zones shown in Figure 2. On the basis of screening by HPLC and TLC it was possible to establish that the crude material consisted of two main components corresponding to fractions in zones 4–6 and 9, respectively. Portions of the crude materials were further purified by semipreparative HPLC in two steps to afford highly pure IA and IB (Figure 2c). The two products were considered to be the diastereomers of I containing (*R*)- and (*S*)-malonyltryptophan. Several pieces of evidence support this, including the identical amino acid composition of the two products, clearly separated double bands on TLC (nanoplates) and good separation of approximately equal peaks by HPLC.[3] Figure 2 illustrates the relationship between the chromatographic behavior of the two.

A more elaborate synthetic route was necessary for the synthesis of analogue II. Five suitably protected precursor segments were coupled in a series of fragment condensations to afford the protected tetradecapeptide as shown in Figure 3. The preparation of the retro–inverso modified segment, Nps-Phe-*g*Trp-(*RS*)-mLys-OH (II-6), posed several synthetic challenges. First, the synthesis of a suitable *m*Lys derivative (II-1) had to be designed, since there was no literature precedent for its preparation. The appropriate α-amino-ϵ-hydroxy compound, 1-hydroxy-4-amino-*n*-butane, was converted to the N^{α}-Boc-protected ϵ-chloro precursor, which was then put into

[3] The presence of two main components was apparent at previous stages of the synthesis by chromatographic techniques (HPLC and TLC).

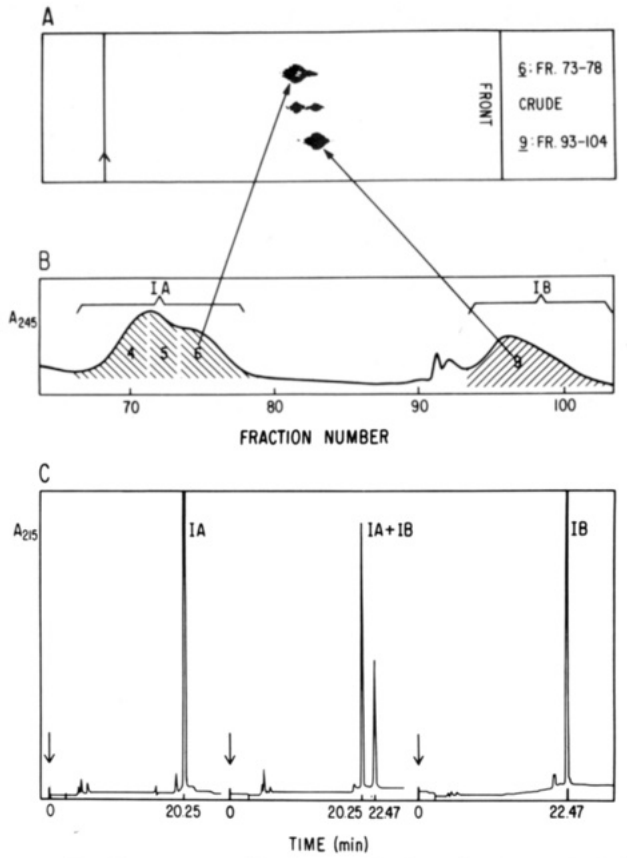
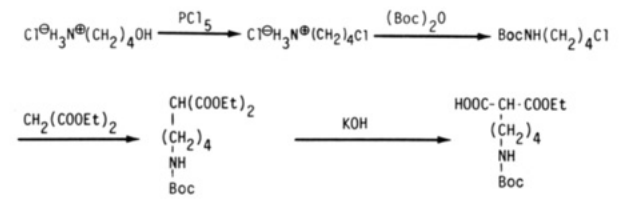


FIGURE 2: Chromatographic characterization of analogue I. (A) HPTLC of the two major fractions of analogue I and the crude material in the solvent system 1-BuOH–AcOH–pyridine–H_2O (30:10:15:20). (B) Purification of analogue I on a Sephadex LH-20 column eluted with 0.1 M NH_4OAc, pH 6.0, buffer. Hatched areas indicate fractions containng the two main products IA and IB. (C) Analytical HPLC of IA and IB after further purification by semipreparative HPLC. The mixture of the two is shown at center. Solvent system i, 30 → 60% B in 30 min.

Scheme I



the malonyl ester reaction to give the diester and finally the monoester of *m*Lys, as shown in Scheme I.

Coupling to a suitable precursor of the *gem*-diaminotryptophan was the next step. This precursor would be L-tryptophanamide if the D-*gem*-diaminoalkyl compound was the desired product and a D-tryptophanamide if the L-*gem*-diamino compound was the target. Note that an amino acid amide in that position is "retro–inverso" (i.e., the $CONH_2$ of the amino acid will become the N-terminal amine while the original amino group will be the "inverso" amino group of the *gem*-diaminoalkyl residue, and therefore, the chirality of the precursor amino acid used has to be opposite to that of the product) (Pallai et al., 1983b). Another problem to overcome was that the indole of tryptophan would not survive the oxidative conditions of the TIB-mediated reaction. Our solution to this problem was the use of the N^{in}-For group which effectively prevented oxidative damage to the indole ring (Pallai et al., 1983b). Thus, the *m*Lys derivative was coupled to D-Trp(For)-NH_2 (II-2) to give the dipeptide derivative II-3

Table I: Biological Activity of Retro–Inverso Modified Somatostatin Analogues[a]

analogue		inhibition of growth hormone release in vitro[b]
gPhe^7-(*RS*)-mTrp^8–SS	IA	0.12 (0.09–0.17)
	IB	0.75 (0.49–1.15)
gTrp^8-(*RS*)-mLys^9–SS	IIA	0.009 (0.006–0.013)
	IIB	0.017 (0.011–0.026)

[a] Relative to somatostatin activity (=1.0). [b] Measured as described previously (Vale et. al., 1972).

which was then converted to the *gem*-diamine II-4 by treatment with TIB. Coupling with Nps-Phe-OH and saponification resulted in the retro–inverso segment II-6 suitable for fragment condensation. Salt coupling, which reduced the number of synthetic steps, was utilized in the synthesis of the protected dipeptide segment II-7 and tripeptide II-13. A series of fragment condensations gave the fully protected 14-peptide which showed the characteristic twin bands on TLC, suggesting the presence of diastereomers. Deprotection and cyclization steps were carried out in the same manner as described for analogue I. The crude cyclized material was purified on the Sephadex LH-20 column as shown in Figure 4. Screening of the fractions indicated that the separation of diastereomers was successful in this case as well as gave the two products IIA and IIB.

The two diastereomers had identical amino acid compositions and showed the characteristic chromatographic pattern (Figure 4) reminiscent of that of other diastereomers of retro–inverso somatostatin and enkephalin analogues (Pallai et al., 1983b). The finding that the two product were interconvertible under conditions which favor the exchange of the α proton of the 2-alkylmalonyl residue further supports the diasteromeric nature of the products.

The two diastereomers of each analogue were tested for growth hormone release inhibiting activity in vitro, and the results are shown in Table I. The diastereomers of both analogues, IA vs. IB and IIA vs. IIB, exhibited significantly different activity which are in accordance with structure–activity findings for the corresponding diastereomers of the native somatostatin: i.e., D-Trp^8-somatostatin has much higher activity than somatostatin (Rivier et al., 1975; Meyers et al., 1978) and D-Lys^6-somatostatin much lower than the native molecule (Vale et al., 1978).

The most intriguing finding is the high activity of analogues IA and IB relative to that of IIA and IIB. This indicates the sensitivity and tolerance of the altered regions to this type of structural modifications; i.e., the Phe–Trp segment is more tolerant than the Trp–Lys segment. A possible explanation for this result may be found when one considers the relative tendencies of these analogues to form a β-turn centered at Trp^8–Lys^9. A type II′ β-turn at this position has been implicated as being important for biological activity (Veber, 1981). Active analogue I, with gPhe at position 8, eliminates the possible stabilizing influence of a 4 → 1 hydrogen bond. However, conformational energy studies of retro–inverso model peptides (Stern et al., 1982) indicate that while the malonyl residue qualifies for the $i + 1$ position of a type II′ β-turn, as is the case in active analogue I, this conformation is highly unfavorable for the *gem*-diamine which occupies this position in inactive analogue II. Therefore, while it is possible that other explanations exist, these results are consistent with a type II′ β-turn at Trp^8–Lys^9 being important for biological activity. Detailed and structural investigations supported by spectro-

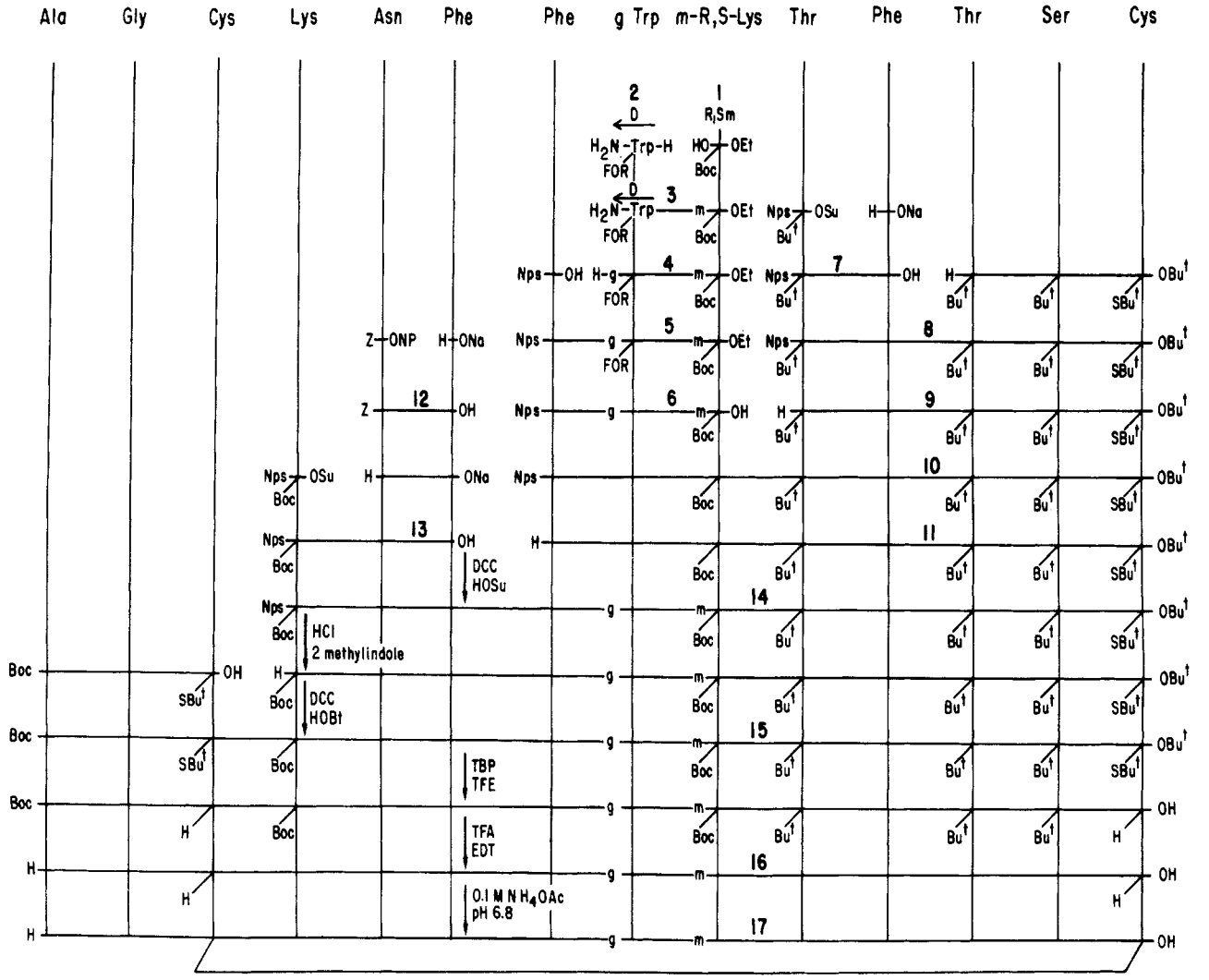


FIGURE 3: Scheme for the synthesis of analogue II.

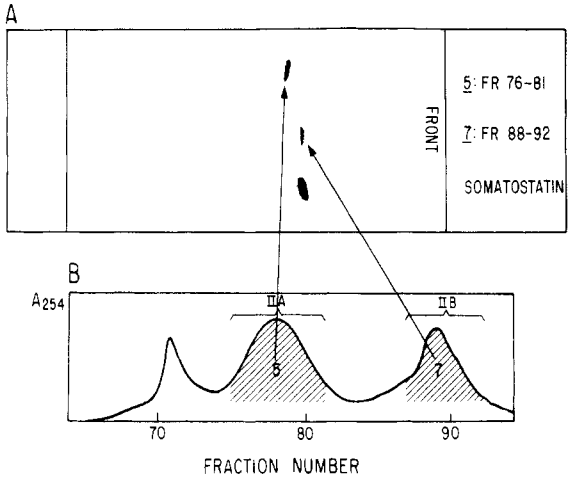


FIGURE 4: Chromatographic characterization of analogue II. (A) HPTLC of the two major fractions of analogue II and the native somatostatin in the solvent system 1-BuOH–AcOH–pyridine–H_2O (30:10:15:20). (B) Purification of analogue II on a Sephadex LH-20 column eluted with 0.1 M NH_4OAc, pH 6.0, buffer. Hatched areas indicate fractions containing the two main products IIA and IIB.

scopic measurements and conformational energy calculations carried out on simpler model systems are more suitable for establishing which conformational features are enhanced or disfavored as a consequence of these modifications (Pallai et al., 1984). This is a focus of our ongoing research.

ACKNOWLEDGMENTS

We thank Jurgen Musiol for his assistance in the deprotection steps, Dr. Paul Thamm for his expert help in the Sephadex LH-20 purification, and Dr. Walter Goehring for the HPLC analyses of chromatographic fractions.

SUPPLEMENTARY MATERIAL AVAILABLE

Description of the synthesis of compounds I-1 and I-5 of analogue I and compounds II-2, II-7, II-8, II-9, II-11, and II-12 of analogue II (4 pages). Ordering information is given on any current masthead page.

Registry No. IA, 95388-54-8; IB, 95463-28-8; I-1, 33900-18-4; I-2, 82653-06-3; I-3, 78220-60-7; I-4, 95388-55-9; I-5, 95388-57-1; I-6, 95388-58-2; I-7, 95388-59-3; I-8, 95388-60-6; I-9, 95388-61-7; I-10, 95388-62-8; IIA, 95463-94-8; IIB, 95388-78-6; II-1, 95388-65-1; II-2, 95388-66-2; II-3, 95463-29-9; II-4, 95463-31-3; II-5, 95388-67-3; II-6, 95388-68-4; II-7, 95388-69-5; II-8, 95388-70-8; II-9, 95388-71-9; II-10, 95388-72-0; II-11, 95388-73-1; II-12, 21467-14-1; II-13, 95388-74-2; II-14, 95388-75-3; II-15, 95388-76-4; II-16, 95388-77-5; [bis(trifluoroacetoxy)iodo]benzene, 2712-78-9; gramine, 87-52-5; diethyl malonate, 105-53-3; Z-Asn-ONp, 3256-57-3; H-Asn-Phe-*g*Phe-(*RS*)-*m*Trp-OH, 95388-63-9; Nps-Lys(Boc)-OSu, 60654-30-0; H-Lys(Boc)-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-Cys(SBu^t)-OBu^t, 77946-28-2; Boc-Ala-Gly-Cys(SBu^t)-OH, 77946-26-0; 4-amino-1-butanol, 13325-10-5; 4-aminochlorobutane hydrochloride, 6435-84-3; *tert*-butyloxycarbonyl anhydride, 24424-99-5; *tert*-butyloxycarbonyl-4-aminochlorobutane, 95388-79-7; Nps-Phe-OH, 2688-22-4; Boc-Phe-OH, 13734-34-4; H-Phe-NH_2, 5241-58-7; Boc-Phe-*g*Phe-

(RS)-mTrp-OH, 95388-80-0; Boc-D-Trp(For)-OH, 64905-10-8; Boc-D-Trp(For)-NH_2, 95388-81-1; H-Phe-OH, 63-91-2; Nps-Thr-(Bu^t)-OSu, 32137-86-3; H-Thr(Bu^t)-Ser(Bu^t)-Cys(SBu^t)-OBu^t·HCl, 77946-22-6.

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Differential Effects of Oxidizing Agents on Human Plasma α_1-Proteinase Inhibitor and Human Neutrophil Myeloperoxidase†

Nancy R. Matheson and James Travis*

Department of Biochemistry, University of Georgia, Athens, Georgia 30602

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ABSTRACT: Human α_1-proteinase inhibitor is easily susceptible to inactivation because of the presence of a methionyl residue at its reactive site. Thus, oxidizing species derived from the myeloperoxidase system (enzyme, H_2O_2, and Cl^-), as well as hypochlorous acid, can inactivate this inhibitor, although H_2O_2 alone has no effect. Butylated hydroxytoluene, a radical scavenger, partially protects α_1-proteinase inhibitor from the myeloperoxidase system and completely protects it from hypochlorous acid. Each oxidant also reacts differently with the inhibitor, in that the myeloperoxidase system and hypochlorous acid can each oxidize as many as six methionyl residues, but hypochlorous acid can also oxidize a single tyrosine residue. Myeloperoxidase can be inactivated by hypochlorous acid, by autoxidation in the presence of H_2O_2 and Cl^-, as well as by H_2O_2 alone. Butylated hydroxytoluene completely protects this enzyme from hypochlorous acid inactivation, does not affect the action of H_2O_2, and enhances autoinactivation. As many as six methionyl residues and two tyrosine residues could be oxidized during autoxidation and six methionine residues by H_2O_2 alone. Eight methionine residues and one tyrosine residue could be oxidized by hypochlorous acid. The tyrosine residue in myeloperoxidase was oxidized only at a relatively high concentration (600 μM) of hypochlorous acid at which point the enzyme simultaneously and completely lost its enzymatic activity. Loss of activity of myeloperoxidase could also be correlated with the loss of the heme groups present in the enzyme when a relatively high concentration of hypochlorous acid (600 μM) was used and also during autoxidation. It appears that once there is sufficient oxidant to modify one of the tyrosine residues, the heme group itself becomes susceptible.

Human α_1-proteinase inhibitor (α_1-PI)[1] is a major circulating plasma protein which is also capable of passing through vascular membranes into tissues during inflammatory episodes. Thus, it is thought to play an important role in controlling tissue proteolysis caused by the massive quantities of proteinases released from polymorphonuclear leukocytes which are attracted to the lungs and joints during inflammation. These

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[1] Abbreviations: α_1-PI, α_1-proteinase inhibitor; MPO, myeloperoxidase; BHT, butylated hydroxytoluene; HOCl, hypochlorous acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Me_2SO, dimethyl sulfoxide.

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