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Structure–Function Studies on Gastrointestinal Hormones

I. Synthesis of Secretin Analogs and Their Biological and Immunological Properties[1,2,3]

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Syntheses by conventional procedures of the three analogs corresponding to the porcine secretin sequence crossed at position 6 by the N-terminal hexapeptide sequences of VIP, GIP, and glucagon are described, viz., Ala[4],Val[5]-, Tyr[1],Ala[2],Glu[3]-, and Gln[3]-secretin (VIP-SN, GIP-SN, and GLU-SN). The analog Phe[1],Phe[2],Trp[3],Lys[4]-secretin (SOMA-SN), designed on the basis of the surprising homology of the sequence portions 10–13 of somatostatin and 5–8 of secretin, was also prepared. Finally, the synthesis of N^{α}-3-(4-hydroxyphenyl)propionyl-β-alanyl-secretin (DATA-SN), a tracer suitable for secretin radioimmunoassay and as an N-terminus modified secretin analog, is reported. The analogs are compared, in terms of their biological and immunological properties in different assay systems, with pure synthetic secretin.

INTRODUCTION

In a previous communication (*1*) we extensively discussed our approach to rationalization of synthetic analog design for structure–function studies on the gastrointestinal hormones. This approach relies upon the specific information

[1] This paper is dedicated to the memory of Professor George W. Kenner in appreciation of his outstanding achievements as a scientist and teacher.

[2] For preliminary communications of some of the results presented in this paper see Refs. (*1–5*).

[3] All amino acids except glycine are of the L-configuration. Standard abbreviations as recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **27**, 201–207 (1972)) are used. Additional abbreviations used: MeOH, methanol: EtOH, ethanol; BuOH, 1-butanol; Et_2O, diethylether; EtOAc, ethyl acetate; DMF, dimethylformamide; DMA, dimethylacetamide; THF, tetrahydrofuran; AcOH, acetic acid; HMPA, hexamethylphosphorictriamide; DCC, dicyclohexylcarbodiimid; HONSu, *N*-hydroxysuccinimide; HOBt, 1-hydroxybenzotriazole; Et_3N, triethylamine; DBSI, dibenzenesulfonimide; cyclic AMP, adenosine 3′ : 5′-monophosphate.

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inherent in the chemical structure of closely related members of the evolution-dependent glucagon family, i.e., glucagon, vasoactive intestinal peptide (VIP), gastric inhibitory peptide (GIP), and secretin. Even though strictly related in regard to their primary structures, the four hormones exhibit differentiated biological properties. A first set of secretin analogs was planned by crossing the secretin molecule at the invariant position 6 with the N-terminal hexapeptide sequences of VIP, GIP, and glucagon, respectively, i.e., Ala[4],Val[5]-, Tyr[1],Ala[2],Glu[3]-, and Gln[3]-secretin (VIP-SN, GIP-SN, and GLU-SN).

The striking homology of the secretin sequence 5–8 and of the somatostatin sequence 10–13 led Robberecht *et al.* (*6*) to speculate on a possible correlation between some properties of somatostatin, especially those observed in the exocrine pancreas, and its chemical structure. This hypothesis prompted us to synthetize Phe[1],Phe[2],Trp[3],Lys[4]-secretin (SOMA-SN), a secretin analog which contains as the N-terminal octapeptide sequence the sequence 6–13 of somatostatin. Furthermore, in order to prepare a suitable tracer for secretin radioimmunoassay (*2–4*) and also to gauge the importance of the N-terminal free amino group for the hormonal activity of secretin, N^{α}-3-(4-hydroxyphenyl)propionyl-β-alanyl-secretin (DATA-SN)[8] was synthesized, wherein the 4-hydroxyphenyl moiety was farther removed from the histidine side chain in order to avoid as much as possible aromatic side chain interactions.

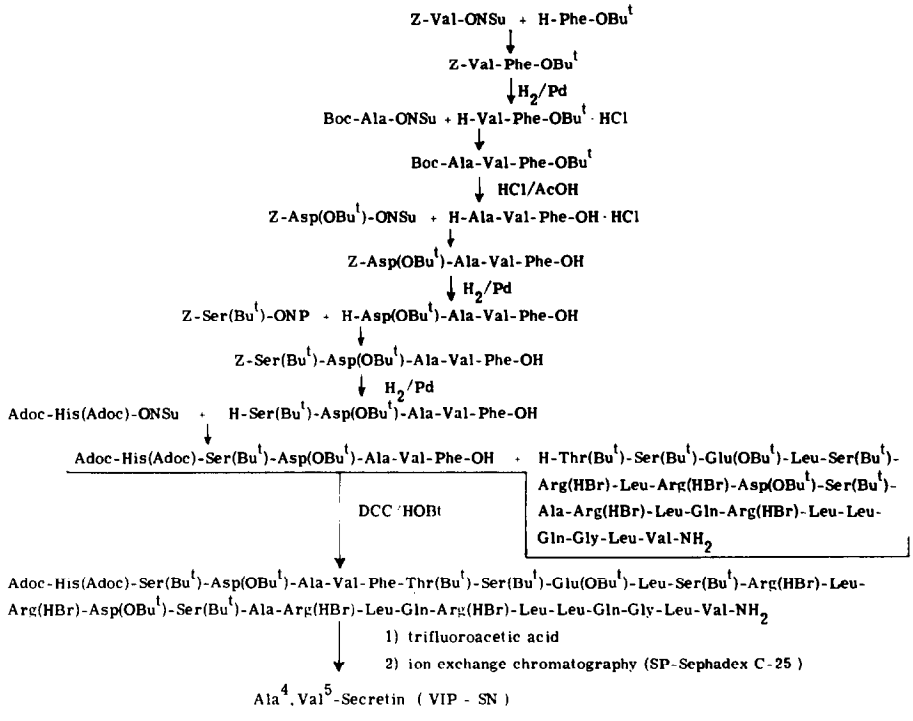
In the present studies the biological and immunological properties of these analogs were determined by different assay systems and compared with those of the parent hormone secretin. In this way, we attempted to reveal specific sequence-dependent information required for the secretin binding affinity to the receptors and full hormonal expression.

In the meantime, the preparation of several somewhat similar secretin analogs has been reported from other laboratories in an effort to bypass the well-known instability of secretin in aqueous solutions (*7–8*) and to secure radioiodinable derivatives for a secretin radioimmunoassay (*9*). On the other hand, charge–charge interactions as stabilizing factors for ordered structures of secretin were investigated by M. Bodanszky, through synthesis of position 9 and 15 analogs (*10*).

SYNTHESIS

The syntheses of the above analogs, all with modifications in the N-terminal region of the secretin molecule, were planned according to the synthetic route successfully elaborated for the total synthesis of secretin (*11*), i.e., by coupling of the suitably protected eneicosapeptide H-Thr(Bu[t])-Ser(Bu[t])-Glu(OBu[t])-Leu-Ser(Bu[t])-Arg(HBr)-Leu-Arg(HBr)-Asp(OBu[t])-Ser(Bu[t])-Ala-Arg(HBr)-Leu-Gln-Arg(HBr)-Leu-Leu-Gln-Gly-Leu-Val-NH_2, H-[7–27]-NH_2 (*11*) with the protected hexapeptide derivatives corresponding to the sequences 1–6 of VIP, GIP, and glucagon, to the sequence 6–13 of somatostatin, and to N^{α}-3-(4-hydroxyphenyl) propionyl-β-alanyl-[1–6] of secretin, respectively.

[8] DATA- = *D*es-*A*mino-*T*yrosyl-β-*A*lanyl-.



SCHEME 1. Synthetic route to Ala^4,Val^5-secretin (VIP-SN).

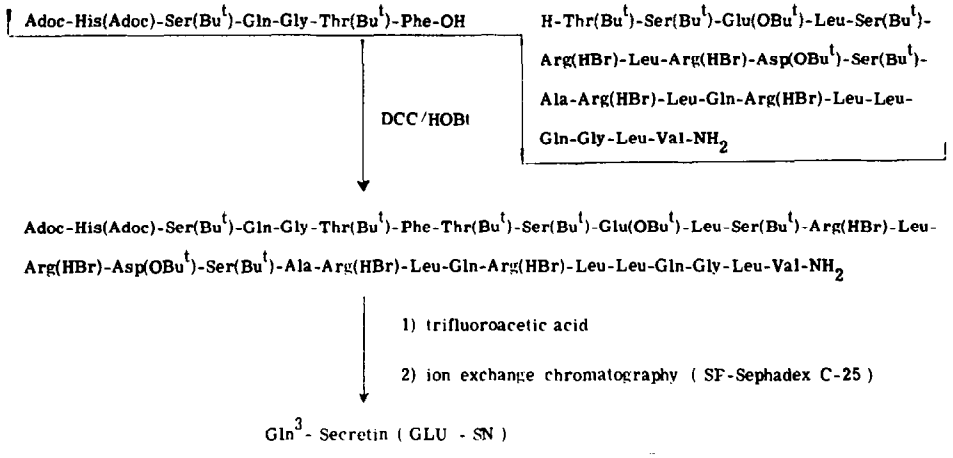
Ala^4,Val^5-Secretin (VIP-SN)

The hexapeptide derivatives Adoc-His(Adoc)-Ser(Bu^t)-Asp(OBu^t)-Ala-Val-Phe-OH corresponding to the N-terminal sequence 1–6 of VIP was synthesized in stepwise manner as shown in Scheme 1. The slow acylation of H-Asp(OBu^t)-Ala-Val-Phe-OH with *Z*-Ser(Bu^t)-ONp[9] was markedly enhanced by adding 1-hydroxybenzotriazole as catalyst (*12*). The pentapeptide, isolated in good yield as homogeneous material, was subsequently debenzyloxycarbonylated by catalytic hydrogenolysis. Its acylation with Adoc-His(Adoc)-ONSu was performed immediately prior to use of the resulting hexapeptide derivative, because of the observed instability of the N^{im}-protection during longer storage periods. After coupling of the hexapeptide derivative with H-[7–27]-NH_2 via DCC/HOBt (*13*), the isolated crude product was exposed to anhydrous trifluoroacetic acid for final deprotection. The purification of the crude synthetic material was achieved by ion exchange chromatography on SP-Sephadex G-25 using ammonium acetate buffer (pH 7.1) at increasing molarity as elutant. Ala^4,Val^5-secretin was obtained as homogeneous material.

[9] The amino acid derivatives used in the synthetic work were prepared according to the procedures reported in Houben-Weyl, "Methoden der Organischen Chemie," (E. Wünsch, Eds.), Vols. 15/I and II, Thieme, Stuttgart, 1974.

Gln³-Secretin (GLU-SN)

The previously described, suitably protected hexapeptide derivative Adoc-His(Adoc)-Ser(Bu^t)-Gln-Gly-Thr(Bu^t)-Phe-OH (*14*) corresponding to sequence 1–6 of glucagon was condensed with the secretin eneicosapeptide H-[7–27]-NH_2 by DCC/HOBt procedure (Scheme 2). Exposure of the resulting crude heptacosapeptide derivative to anhydrous trifluoroacetic acid, followed by ion exchange



SCHEME 2. Synthetic route to Gln³-secretin (GLU-SN).

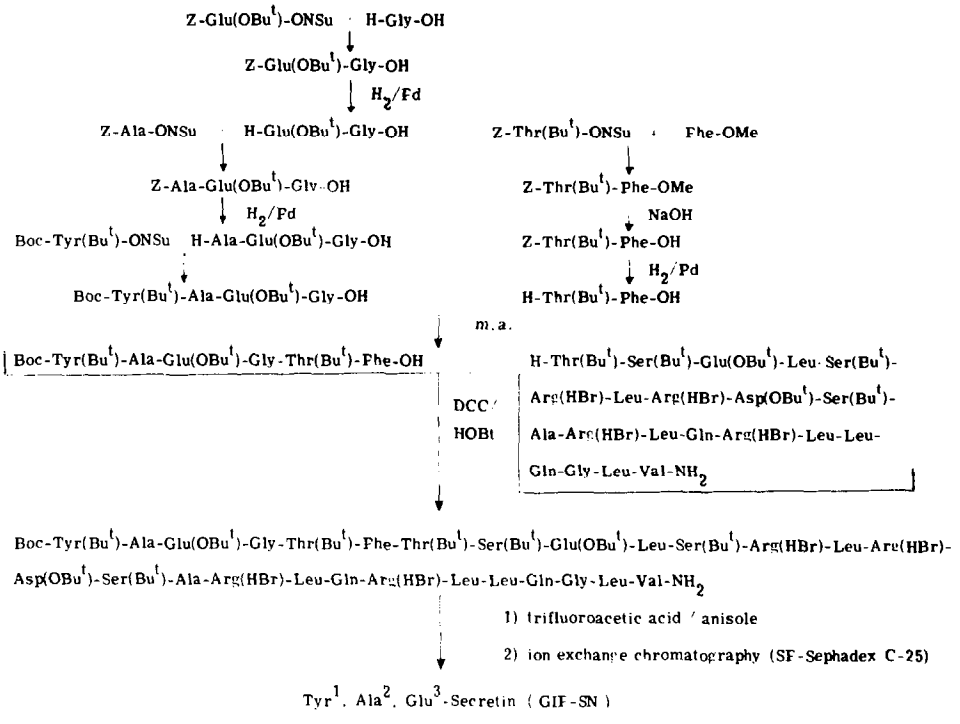
chromatography on SP-Sephadex G-25 using ammonium acetate buffer at different pH and molarity values as eluant, led to the Gln³-secretin, homogeneous as judged by various analytical tests.

Tyr¹,Ala²,Glu³-Secretin (GIP-SN)

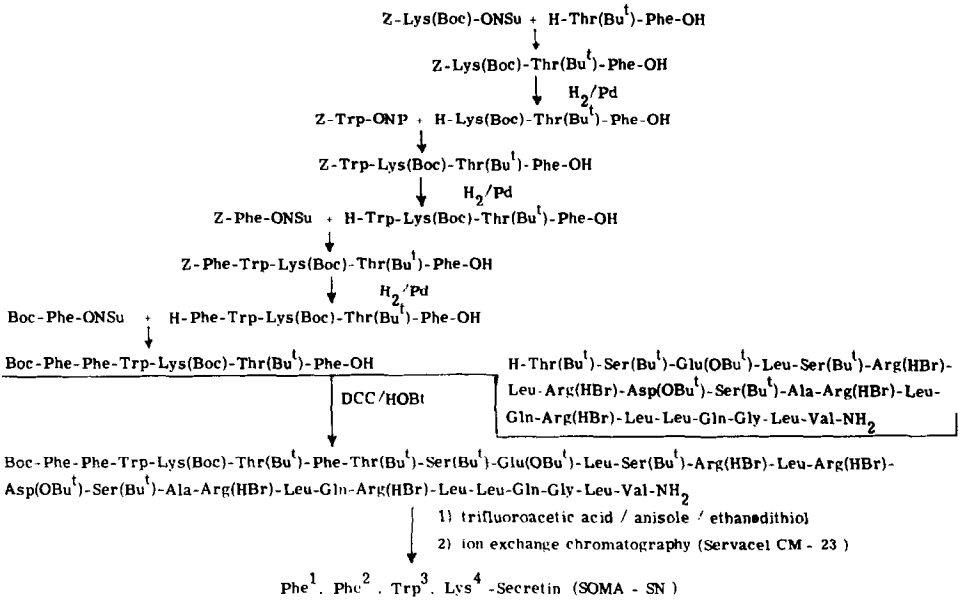
The hexapeptide Boc-Tyr(Bu^t)-Ala-Glu(OBu^t)-Gly-Thr(Bu^t)-Phe-OH (sequence 1–6 of GIP) was obtained in satisfactory yield and homogeneity by mixed anhydride coupling of the tetrapeptide derivative Boc-Tyr(Bu^t)-Ala-Glu(OBu^t)-Gly-OH (prepared in stepwise manner using exclusively *N*-hydroxysuccinimido esters as acylating agents) with the previously reported (*12*) dipeptide derivative H-Thr(Bu^t)-Phe-OH (Scheme 3). The final coupling step to produce the protected heptacosapeptide derivative, its deprotection, and purification was performed according to the above-mentioned procedures. Tyr¹,Ala²,Glu³-secretin was obtained as homogenous material, within the limits of error of the analytical procedures used.

Phe¹,Phe²,Trp³,Lys⁴-Secretin (SOMA-SN)

The suitably protected hexapeptide derivative corresponding to the sequence 6–13 of somatostatin was synthesized as shown in Scheme 4, involving stepwise acylation procedures via *N*-hydroxysuccinimido and 4-nitrophenyl esters, whereby special attention was paid to all reaction steps of the tryptophan-



SCHEME 3. Synthetic route to Tyr^1,Ala^2,Glu^3-secretin (GIP-SN).



SCHEME 4. Synthetic route to Phe^1,Phe^2,Trp^3,Lys^4-secretin (SOMA-SN).

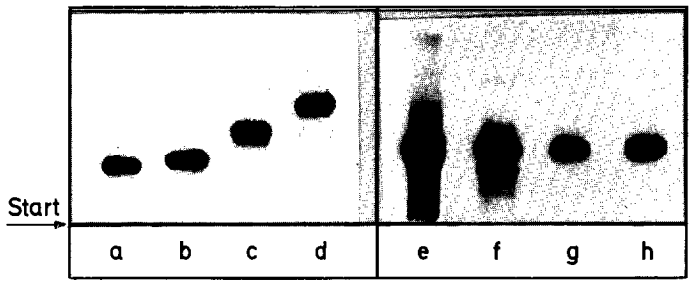
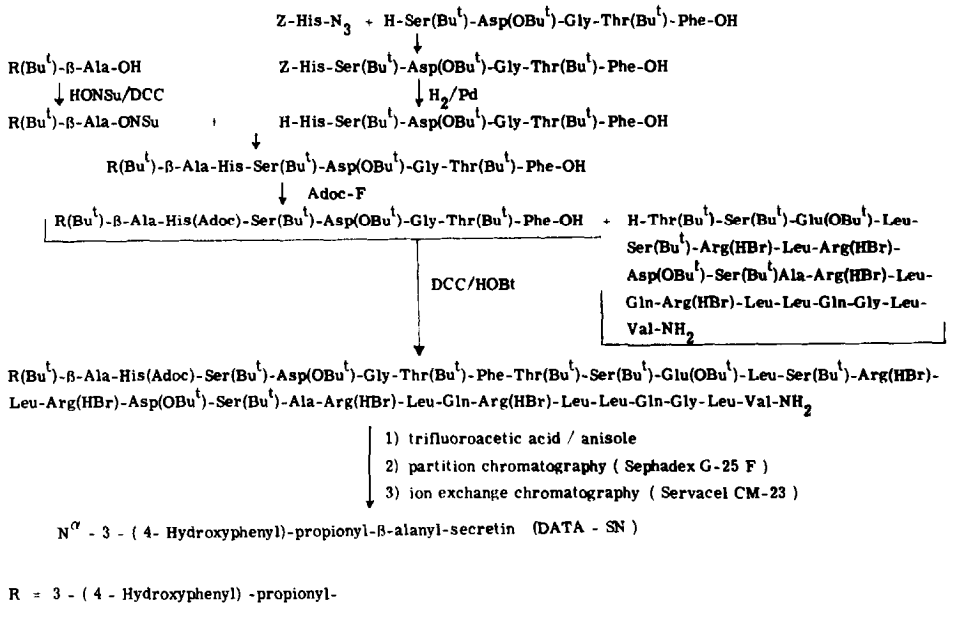


FIG. 1. Thin-layer chromatogram of the synthetic secretin analogs on silica gel plates: solvent system 6. Staining with chlorine–*p*-tolidine method (*20*); (a) Gln^3-secretin (GLU-SN); (b) Ala^4,Val^5-secretin (VIP-SN); (c) Tyr^1,Ala^2,Glu^3-secretin (GIP-SN); (d) Phe^1,Phe^2,Trp^3,Lys^4-secretin (SOMA-SN); (e–h) N^{α}-3-(4-hydroxyphenyl)propionyl-β-alanyl-secretin (DATA-SN): (e) crude product XLII-A; (f) fraction XLII-B_1 from ion exchange chromatography; (g) fraction XLII-C_1 from partitition chromatography; (h) fraction XLII-D_1 from free-flow electrophoresis.

containing peptide derivatives, to avoid well-known indole side reactions. The resulting homogeneous hexapeptide derivative Boc-Phe-Phe-Trp-Lys(Boc)-Thr(Bu^t)-Phe-OH was subsequently condensed with H-[7–27]-NH_2 via DCC/HOBT to give the heptacosapeptide derivative, which was deprotected by exposure to anhydrous trifluoroacetic acid in the presence of anisole and ethandithiol as scavengers. Ion exchange chromatography on Servacel CM-23 using ammonium acetate buffer (pH 7.25) at increasing molarity values as eluant, yielding the desired product in satisfactory homogeneity (Fig. 1).

N^{α}-3-(4-Hydroxyphenyl)propionyl-β-alanyl-secretin (DATA-SN)

The key compound for the synthesis of the title analog, i.e., 3-(4-*tert*-butoxyphenyl)propionic acid *N*-hydroxysuccinimido ester, was prepared by acid-catalyzed etherification of the phenol function of 3-(4-hydroxyphenyl)propionic acid 4-nitrobenzyl ester with isobutene, followed by saponification of the 4-nitrobenzyl ester and activation of the resulting free carboxyl function, according to well-established methods in peptide chemistry. The *N*-hydroxysuccinimido ester was allowed to react with β-alanine in aqueous organic medium, giving homogeneous 3-(4-*tert*-butoxyphenyl)propionyl-β-alanine, subsequently converted into the corresponding *N*-hydroxysuccinimido ester. An attempted preparation of the suitably protected hexapeptide derivative 1–6 of secretin, H-His(Adoc)-Ser(Bu^t)-Asp(OBu^t)-Gly-Thr(Bu^t)-Phe-OH via acylation of H-Ser(Bu^t)-Asp(OBu^t)-Gly-Thr(Bu^t)-Phe-OH (*15*) with *Z*-His(Adoc)-ONSu followed by hydrogenolytic decarbobenzoxylation, failed because of the extreme instability of the N^{im}-acyl derivative during the N^{α}-deprotection and subsequent workup. Thus the route shown in Scheme 5 was planned and reduced to practice, in which the imidazole-unprotected hexapeptide derivative H-His-Ser(Bu^t)-Asp(OBu^t)-Gly-Thr(Bu^t)-Phe-OH was acylated by 3-(4-*tert*-butoxyphenyl)propionyl-β-Ala-ONSu in good yields. The resulting homogeneous, stable derivative was subsequently blocked at the histidine imidazole function by direct acylation (*4*, *16*) via Adoc-fluoride (*17*). Thus any imidazole-mediated side reaction during the final coupling



SCHEME 5. Synthetic route to N^{α}-3-(4-hydroxyphenyl)propionyl-β-alanyl-secretin (DATA-SN).

of the hexapeptide derivative to H-[7–27]-NH_2 is prevented. This condensation was performed via DCC/HOBt, and the resulting heptacosapeptide derivative was fully deprotected by exposure to anhydrous trifluoroacetic acid containing 10% anisole as scavenger. Preliminary attempts to purify the crude product led, after SP-Sephadex C-25 ion exchange chromatography using ammonium acetate buffers (pH 6.0) with increasing molarities as eluant, followed by partition chromatography on Sephadex G-25F (solvent system: 2-BuOH–0.05/*M*/AcOH–EtOH, 1 : 1 : 0.05 v/v; pH 3.4), or free flow electrophoresis (Fig. 2), to the products XLII-

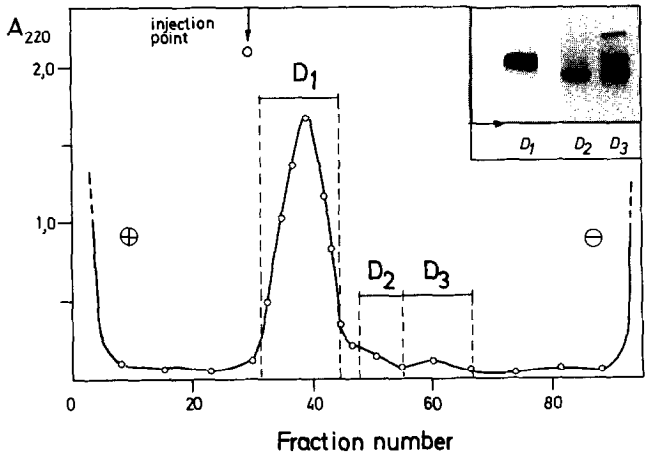


FIG. 2. Free-flow electrophoresis of DATA-SN fraction XLII-C_1 (see Experimental): peptide distribution and thin-layer chromatogram in solvent system 6 on silica gel plate of the fractions D_1 (= pure DATA-secretin; XLII-D_1), D_2, and D_3 (impurities separated from D_1).

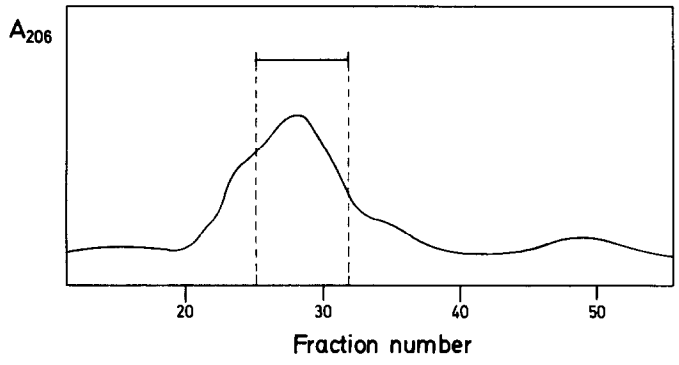


FIG. 3. Partition chromatography of crude N^{α}-3-(4-hydroxyphenyl)propionyl-β-alanyl-secretin (DATA-SN) on Sephadex G-25F (see Experimental); elution profile. Fractions collected are indicated by the horizontal bar.

C_1 and XLII-D_1, respectively. Both preparations correspond, within the limits of detection of the analytical procedures used, to the desired N^{α}-3-(4-hydroxyphenyl)propionyl-β-alanyl-secretin. A simpler purification procedure was finally evolved, based on partition chromatography on Sephadex G-25 (solvent system: 2-BuOH–0.05/*M*/AcOH–EtOH, 1:1:0.05 v/v; pH 4.5), followed by the ion exchange chromatography on Servacel CM-23 with ammonium acetate buffers at increasing pH and molarity values as eluants (Figs. 3 and 4). This procedure produced the desired material in good yields (52%) with chromatographic and electrophoretic properties identical to those of the compounds XLII-C_1 and D_1 (Fig. 1).

The purity of the synthetic analogs was thoroughly checked, not only by tlc, but also by means of a special electrophoretic technique. Due to the high isoelectric point of secretin (approximately 11) and its analogs, focusing cannot be performed very easily. Therefore the usual isoelectric focusing system was used to carry out

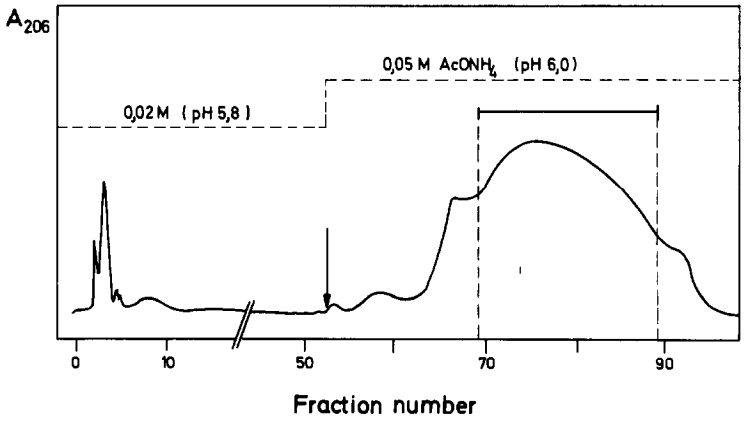


FIG. 4. Ion exchange chromatography of prepurified (see Fig. 3) DATA-SN on Servacel CM-23 (see Experimental); elution profile. Fractions collected are indicated by the horizontal bar.

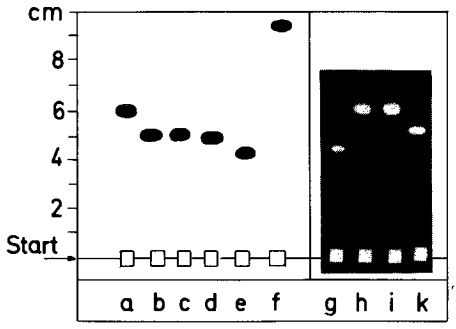


FIG. 5. Analytical electrophoresis of the synthetic secretin analogs on Ampholine-PAG plates (pH 3.5–9.5); for electrophoretic conditions and visualization see Experimental. (a) Gln^3-secretin (GLU-SN), (b) Ala^4,Val^5-secretin (VIP-SN), (c) secretin, (d) Tyr^1,Ala^2,Glu^3-secretin (GIP-SN), (e) N^{α}-3-(4-hydroxyphenyl)propionyl-β-alanyl-secretin (DATA-SN), (f) cytochrome *c* as marker, (g) crude DATA-SN (350 μg), (h) crude GLU-SN (300 μg), (i) pure GLU-SN (360 μg), (k) crude VIP-SN (420 μg).

a type of electrophoresis in a definite pH gradient without attempting to focus these peptides. The system responds very well to charge differences between secretin and its analogs, as shown in Fig. 5(a–e). As an example, Fig. 5 shows several bands of impurities for the crude DATA-SN (g), GLU-SN (h), and VIP-SN (k), while one single band underlines the homogeneity of purified GLU-SN (i).

EXPERIMENTAL

Unless stated otherwise the reactions were conducted at room temperature and the solvents evaporated under reduced pressure at a bath temperature below 40°C in a rotatory evaporator. The compounds were dried *in vacuo* at 40–50°C over KOH pellets. The elemental analyses were performed in the microanalytical laboratory of the department on Perkin–Elmer 240 Elemental Analyzers.

Hydrogenations were routinely conducted in the indicated solvent at room temperature and atmospheric pressure. Concomitant titrations of the free amino function in the specified cases were performed with the Dosimat E 35 (Metrohm, Herisau, Switzerland). All solvents were of reagent grade and were distilled before use. 2-Butanol was purified according to the procedure described previously (*18*). Amino acids were purchased from Fluka AG (Buchs, Switzerland).

Thin-layer chromatography was performed on silica gel plates 60 (Merck AG, Darmstadt) in the following solvent systems: 2, BuOH–AcOH–H_2O (3 : 1 : 1); 6, BuOH–AcOH–H_2O–pyridine (60 : 6 : 24 : 20); 23, isoamyl alcohol–pyridine–H_2O (35 : 35 : 30); 27, *n*-heptane–*tert*-butanol–AcOH (3 : 2 : 1); 40b, $CHCl_3$–MeOH (5 : 1); 46, MeOH–AcOH–CH_2Cl_2 (1 : 2 : 3). Compounds were visualized on thin-layer plates by fluorescamine (*19*) and the chlorine reagent (*20*). Unless stated otherwise the compounds were chromatographically homogeneous in the reported solvent systems. Melting points were determined on a Tottoli's capillary melting point apparatus and were uncorrected. Optical rotations were measured on a

Perkin–Elmer 241 MC polarimeter. The acid hydrolyses were conducted in 6 *N* HCl at 110°C for 20 hr (unless stated otherwise) adding thioglycolic acid for Trp-containing peptides (*21*) in evaculated, sealed ampoules. Amino acid analyses were obtained on the Beckman amino acid analyzer with automatic digital integrator (Models 120B and 125). The peptide content of lyophilized materials was determined by quantitative amino acid analysis on the basis of the molecular weight of the title compound formula. The yield (%) of lyophilized material was determined on the basis of the peptide content.

SP-Sephadex C-25 (Na^+ form) and Sephadex G-25F were purchased from Pharmacia (Freiburg) and Servacel CM-23 (H^+ form) from Serva (Heidelberg). Peptides after column chromatography were detected by monitoring the uv adsorption of the solution using LKB-Uvicord III. Ultraviolet measurements were carried out on a Cary Model 118 C recording spectrophotometer. Elphor VaP_2 free-flow electrophoresis apparatus (Bender and Hobein, München) was modified for fractionation in 95 tubes.

Analytical electrophoreses on thin-layer polyacrylamide gel with a pH gradient of 3.5–9.5 (Ampholine-PAG plates, LKB) were performed at 7°C in a Multiphor electrophoresis system (LKB 2117) with a LKB 3371 E power supply. The samples, dissolved in 10% sorbitol solutions, were applied by means of pieces of filter paper (Whatman 3MM). Electrode solutions were used in accordance to the manufacturers' instructions. Electrophoreses were carried out for 1 hr 20 min starting with 220 V and increasing the voltage every 10 min until 900 V. After the run, peptides were precipitated by immersing the gel in a 12.5% solution of trichloroacetic acid; precipitated bands could be visualized and photographed without staining.

Ala^4, *Val*^{5}-*Secretin*

Z-Val-Phe-OBu^t(*I*). Z-Val-ONSu (35 g, 0.1 mol) was added to a solution of H-Phe-OBu^t, DBSI (52 g, 0.1 mol), Et_3N (14 ml, 0.1 mol), and pyridine (8 ml) in DMF (500 ml), and the reaction was allowed to proceed for 72 hr under stirring. The solvent was evaporated, the residue dissolved in EtOAc and extracted with 1 *N* HCl, 1 *N* Na_2CO_3, and H_2O. The solution was dried over Na_2SO_4 and evaporated to dryness. The product was crystallized from EtOAc–petroleum ether; yield: 43 g (95%); mp 102–103°C; $[\alpha]_D^{20}$ − 31.8° and $[\alpha]_{546}^{20}$ − 38.2° (c 1, MeOH); tlc: 36.

Anal. Calcd. for $C_{26}H_{34}N_2O_5$ (454.6): C, 68.70; H, 7.54; N, 6.6. Found: C, 68.59; H, 7.56; N, 6.28.

H-Val-Phe-OBu^t · *HCl* (*II*). The protected dipeptide I (42 g, 92.5 mmol) was hydrogenated in MeOH (1.5 liter) at pH 5 over Pd-black titrating with 2 *N* HCl in MeOH. The catalyst was removed by filtration and the filtrate evaporated. The residue was solidified by addition of Et_2O, collected, and washed extensively with Et_2O; yield: 31.5 g (95%); mp 197–199°C; $[\alpha]_D^{20}$ +43.6° and $[\alpha]_{546}^{20}$ +54.0° (c 1, DMF); tlc: 36.

Anal. Calcd. for $C_{18}H_{29}N_2O_3Cl$ (356.9): C, 60.58; H, 8.19; N, 7.85; Cl, 9.93. Found: C, 60.52; H, 8.38; N, 7.79; Cl, 10.48.

Boc-Ala-Val-Phe-OBu^t (*III*). To a suspension of compound II (59 g, 0.165 mol)

in CH_2Cl_2 (1.5 liters) Et_3N (23.1 ml, 0.165 mol) was added, followed by Boc-Ala-ONSu (45.8 g, 0.160 mol). After 48 hr stirring, the bulk of the solvent was evaporated and the residue distributed between EtOAc/H_2O. The organic phase was washed with 1 *N* H_2SO_4, 10% $KHCO_3$, and H_2O, dried over Na_2SO_4, and evaporated. The product was crystallized from EtOAc–Et_2O–petroleum ether; yield: 71 g (90%); mp 153–155°C; $[\alpha]_D^{20}$ +56.39° and $[\alpha]_{546}^{20}$ −68.3 (c 1, MeOH); tlc: 36.

Anal. Calcd. for $C_{26}H_{41}O_3N_6$ (491.64): C, 63.52; H, 8.41; N, 8.55. Found: C, 63.55; H, 8.34; N, 8.73.

H-Ala-Val-Phe-OH · HCl (IV). Compound III (71 g, 0.144 mol) was dissolved in 4 *N* HCl in AcOH. After 1 hr the solvent was evaporated and the solid residue triturated with Et_2O, collected, and washed with Et_2O; yield: 54 g (quantitative); $[\alpha]_D^{20}$ −26.25° and $[\alpha]_{546}^{20}$ −31.33° (c 1.1, 1 *N* HCl); tlc: 2, 6.

Anal. Calcd. for $C_{17}H_{26}N_3O_4Cl$ (371.87): C, 54.91; H, 7.05; N, 11.30; Cl, 9.53. Found: C, 54.88; H, 7.04; N, 11.10; Cl, 9.37.

Z-Asp (OBu^t)-Ala-Val-Phe-OH (V). *Z*-Asp(OBu^t)-ONSu (21.9 g; 60 mmol) in dioxane (300 ml) was added to a solution of the tripeptide IV (18.6 g, 50 mmol) and $KHCO_3$ (6.0 g, 60 mmol) in 0.5 *N* NaOH (200 ml) and dioxane (100 ml). The reaction mixture was stirred for 72 hr and then acidified to pH 4 with 1 *N* H_2SO_4 (110 ml). The precipitate was collected and washed extensively with H_2O; yield: 29 g (90.5%); mp 193°C; $[\alpha]_D^{20}$ −25.83° and $[\alpha]_{546}^{20}$ −30.91° (c 1.5, EtOH); tlc: 36, 27.

Anal. Calcd. for $C_{33}H_{44}N_4O_9$ (640.7): C, 61.86; H, 6.92; N, 8.74. Found: C, 61.70; H, 6.92; N, 8.56.

H-Asp(OBu^t)-Ala-Val-Phe-OH (VI). The protected tetrapeptide V (25.6 g, 39.9 mmol) was hydrogenated in AcOH (500 ml) for 2 hr over Pd-black. The catalyst was filtered off and the filtrate evaporated to a small volume. On addition of EtOAc the precipitate was collected by filtration and washed with MeOH; yield: 18.8 g (93%); $[\alpha]_D^{20}$ −11.7° and $[\alpha]_{546}^{20}$ −13.9° (c 1, AcOH); tlc: 36, 2.

Anal. Calcd. for $C_{25}H_{38}N_4O_7$ (506.6): C, 59.27; H, 7.56; N, 11.06. Found: C, 58.98; H, 7.61; N, 11.05.

Z-Ser(Bu^t)-Asp(OBu^t)-Ala-Val-Phe-OH (VII). To a suspension of compound VI (22.6 g, 44.6 mmol) in pyridine (750 ml) Et_3N (6.24 ml, 44.6 mmol), HOBt (6.03 g, 44.6 mmol), and *Z*-Ser(Bu^t)-ONp (27.8 g, 66.9 mmol) were added. A clear solution was formed after 10 min and the reaction was allowed to proceed for additional 24 hr. The bulk of the solvent was evaporated and the residue triturated with Et_2O. The solid was collected, washed with Et_2O, and air-dried. Its solution in MeOH was acidified to pH 3 with 1 *N* HCl and immediately diluted with H_2O. The precipitate was collected, washed with H_2O, and crystallized from MeOH–H_2O; yield: 25 g (71.6%); mp 217°C; $[\alpha]_D^{20}$ −30.2° and $[\alpha]_{546}^{20}$ −36.4° (c 1, MeOH); tlc: 2, 6.

Anal. Calcd. for $C_{40}H_{57}N_5O_{11}$ (783.9): C, 61.29; H, 7.33; N, 8.93. Found: C, 61.07; H, 7.45; N, 8.87.

H-Ser(Bu^t)-Asp(OBu^t)-Ala-Val-Phe-OH (VIII). The protected pentapeptide VII (2.6 g, 3.3 mmol) was hydrogenated in MeOH (150 ml) over Pd-black at pH 4 by titrating the solution with 0.5 *N* HCl. The catalyst was removed and the filtrate neutralized with Et_3N (0.455 ml). On standing in the cold the product crystallized;

it was filtered off and washed with MeOH and Et_2O; yield: 1.76 g (82%); $[\alpha]_D^{20}$ −18.6° and $[\alpha]_{546}^{20}$ −22.4° (c 0.85, AcOH); tlc: 2, 40b.

Anal. Calcd. for $C_{32}H_{51}N_5O_9$ (649.79): C, 59.15; H, 7.91; N, 10.78. Found: C, 59.0; H, 7.96; N, 10.85.

Adoc-His(Adoc)-Ser(Bu^t)-Asp(OBu^t)-Ala-Val-Phe-OH (IX). The partially protected hexapeptide VIII (2.0 g, 3.07 mmol) was dissolved in MeOH (100 ml) by addition of 2.05 *N* benzyltrimethylammonium hydroxide in MeOH (1.5 ml, 3.07 mmol). The solution was evaporated to dryness and the residue dissolved in DMF (60 ml). At 0°C Adoc-His(Adoc)-ONSu (3.73 g, 6.14 mmol) was added and the reaction mixture was stirred overnight at 4°C and additional 6 hr at room temperature. On addition of 1 *N* HCl (3.1 ml) and H_2O a precipitate was formed, which was collected by centrifugation, washed with EtOAc, and finally reprecipitated from DMF with EtOAc; yield: 2.5 g (71%); tlc: 2, 23, 27, 46.

Anal. Calcd. for $C_{60}H_{86}N_8O_{14} \cdot 2H_2O$ (1179.4): C, 61.1; H, 7.69; N, 9.50. Found: C, 61.15; H, 7.56; N, 9.59.

Adoc - His(Adoc) - Ser(Bu^t) - Asp(OBu^t) - Ala - Val - Phe - Thr(Bu^t) - Ser(Bu^t) - Glu(OBu^t) - Leu - Ser(Bu^t) - Arg(HBr) - Leu - Arg((HBr) - Asp(OBu^t) - Ser(Bu^t) - Ala - Arg(HBr) - Leu - Gln - Arg(HBr) - Leu - Leu - Gln - Gly - Leu - Val - NH_2 (X). To a solution of H - Thr(Bu^t) - Ser(Bu^t) - Glu(OBu^t) - Leu - Ser(Bu^t) - Arg(HBr) - Leu - Arg(HBr) - Asp(OBu^t) - Ser(Bu^t) - Ala - Arg(HBr) - Leu - Gln - Arg(HBr) - Leu - Leu - Gln - Gly - Leu - Val - $NH_2 \cdot$ HBr (0.34 g, 0.107 mmol) and Et_3N (15 μl, 0.107 mmol) in DMF (4 ml), DMA (12 ml), and HMPA (6 ml) the protected hexapeptide IX (0.37 g, 0.32 mmol) was added, followed at −10°C by HOBt (66 mg, 0.45 mmol) and DCC (66 mg, 0.32 mmol). The reaction mixture was stirred 72 hr at 4°C, and 12 hr at room temperature; then additional compound IX (0.122 mg, 0.107 mmol) was added, followed at −10°C by HOBt (20 mg, 0.15 mmol) and DCC (22 mg, 0.107 mmol). The solution was allowed to reach room temperature in 2 hr and was stirred for an additional 24 hr. Insoluble material was removed by filtration and the filtrate poured into EtOAc (250 ml). The precipitate was collected, washed with EtOAc and Et_2O; yield: 0.34 g; tlc: 2 (the product is slightly contaminated by impurities with lower R_f values). Amino acid ratios in acid hydrolysate: His 0.82 (1) Ser 3.93 (4) Asp 2.00 (2) Ala 1.99 (2) Val 1.78 (2) Phe 0.85 (1) Thr 0.90 (1) Glu 2.97 (3) Leu 6.22 (6) Arg 4.23 (4) Gly 1.10 (1).

H-His-Ser-Asp-Ala-Val-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Asp-Ser-Ala-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH_2 (VIP-SN; XI). The protected heptacosapeptide X (0.32 g, 0.077 mmol) was added to ice-cold anhydrous trifluoroacetic acid (10 ml). The resulting solution was kept under argon at room temperature for 150 min and then concentrated to half the volume. Et_2O was added and the precipitate collected by filtration, washed with Et_2O, and dried over KOH pellets. The crude product (0.3 g) was dissolved in 1% AcOH (10 ml) and applied to a SP-Sephadex C-25 column (54 × 2 cm) equilibrated with 0.05 *M* ammonium acetate buffer (pH 6.5) and eluted successively with 0.05 *M* (pH 6.5; 1200 ml) and 0.075 *M* ammonium acetate buffer (pH 7.1) at a flow rate of 75 ml/hr; fractions of 110 ml were collected after the buffer change. The eluction profile (see Ref. (1)) was monitored by continuous spectrophotometric measurements at 206 nm. Fractions 10–23 of the 0.075 *M* eluate were pooled and lyophilized. The

residue was dissolved in 1% AcOH, the solution filtered through a Millipore filter GSTF (0.22 μm) and lyophilized; the filtration and lyophilization procedure was repeated two more times; yield: 148 mg (50%) of fluffy material which appeared homogenous on silica gel tlc (solvent system 6; see Fig. 1) and on "isoelectric focusing" (Fig. 5); amino acid ratios in acid hydrolysate: His 1.01 (1) Ser 3.99 (4) Asp 1.97 (2) Ala 2.01 (2) Val 1.95 (2) Phe 1.02 (1) Thr. 1.02 (1) Glu 3.01 (3) Leu 5.84 (6) Arg 4.02 (4) Gly 1.00 (1); peptide content: 80%.

Gln^3-Secretin

Adoc-His(Adoc)-Ser(Bu^t)-Gln-Gly-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(*Bu^t*)-*Glu(OBu^t)-Leu-Ser(Bu^t)-Arg(HBr)-Leu-Arg(HBr)-Asp(OBu^t)-Ser(Bu^t)-Ala-Arg(HBr)-Leu-Gln-Arg(HBr)-Leu-Leu-Gln-Gly-Leu-Val-NH_2* (*XII*). Adoc-His(Adoc)-Ser(Bu^t)-Gln-Gly-Thr(Bu^t)-Phe-OH (0.367 g), 0.321 mmol) was coupled to H-Thr(Bu^t)-Ser(Bu^t)-Glu(OBu^t)-Leu-Ser(Bu^t)-Arg(HBr)-Leu-Arg(HBr)-Asp(OBu^t)-Ser(Bu^t)-Ala-Arg(HBr)-Leu-Gln-Arg(HBr)-Leu-Leu-Gln-Gly-Leu-Val-$NH_2 \cdot$ HBr (0.34 g, 0.107 mmol) and the product isolated as described for X; yield: 0.33 g; tlc: 2 (the product is contaminated by a trace impurity of lower R_f value); amino acid ratios in the acid hydrolysate: His 0.71 (1) Ser 3.97 (4) Glu 3.89 (4) Gly 2.00 (2) Thr 1.94 (2) Phe 0.97 (1) Leu 5.90 (6) Arg 3.68 (4) Asp 1.02 (1) Ala 1.00 (1) Val 0.94 (1).

H-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Asp-Ser-Ala-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH_2 (*GLU-SN; XIII*). The protected heptacosapeptide XII (0.3 g, 0.072 mmol) was added to ice-cold anhydrous trifluoroacetic acid (10 ml). The resulting solution was kept under argon for 150 min at room temperature. The solid obtained on addition of Et_2O was collected, washed extensively with Et_2O, and dried over KOH pellets. The crude heptacosapeptide amide (0.25 g) was dissolved in 1% AcOH (10 ml) and applied to a SP-Sephadex C-25 column (54 × 2 cm) equilibrated with 0.05 *M* ammonium acetate buffer (pH 6.5). The column was eluted at a flow rate of 75 ml/hr successively with following ammonium acetate buffers: ca. 1700 ml of 0.05 *M* (pH 6.5), ca. 2000 ml of 0.075 *M* (pH 7.1), ca. 1500 ml of 0.075 *M* (pH 7.5), 0.1 *M* (pH 7.63); and 0.11 *M* (pH 7.63); fractions of 100 ml were collected after the third buffer change. By continuous spectrophotometric measurements at 206 nm the elution profile was registered (see Ref. (*1*)). Fractions 12–38 of the 0.1 and 0.11 *M* eluate were collected and the product was isolated by lyophilization as described for XI; yield: 125 mg (44%) of fluffy material, which behaved homogeneously on silical gel tlc (solvent system 6, see Fig. 1) and on "isoelectric focusing" (Fig. 5); amino acid ratios in acid hydrolysate: His 1.01 (1) Ser 4.00 (4) Asp 0.98 (1) Glu 4.00 (4) Gly 1.99 (1) Phe 1.03 (1) Thr 1.97 (2) Leu 5.95 (6) Arg 4.07 (4) Ala 1.01 (1) Val 0.95 (1); peptide content: 78.4%.

Tyr^1,Ala^2,Glu^3-Secretin

Z-Glu(OBu^t)-Gly-OH (*XIV*). To a solution of H-Gly-OH (49.5 g, 0.66 mol) in THF (250 ml) and H_2O (750 ml) containing 4 *N* NaOH (165 ml, 0.66 mol) was added *Z*-Glu(OBu^t)-ONSu (143.5 g, 0.33 mol) in THF (500 ml). After 20 hr stirring, *N*-(2-aminoethyl)-piperazine (5 ml) was added and the mixture stirred for an

additional 45 min. The bulk of THF was evaporated, the aqueous solution acidified with $KHSO_4$ to pH 2 and extracted twice with EtOAc. The combined EtOAc layers were washed with dilute $KHSO_4$ solution and H_2O, dried over Na_2SO_4, and evaporated; yield: 130.5 g oil (quantitative); tlc: 28, 36.

H-Glu(OBu^t)-Gly-OH (XV). The protected dipeptide XIV (130 g, 0.33 mol) was hydrogenated in MeOH (2 liters) and H_2O (1 liter) over Pd-black for 20 hr. The filtrate was evaporated to dryness and the residue crystallized from MeOH; yield: 78 g (90%); mp 181–182°C; $[\alpha]_D^{20}$ +73.0° and $[\alpha]_{546}^{20}$ +87.3° (c 2, H_2O); tlc: 27, 6.

Anal. Calcd. for $C_{11}H_{20}N_2O_5$ (260.29): C, 50.76; H, 7.74; N, 10.76. Found: C, 50.57; H, 7.76; N, 10.68.

Z-Ala-Glu(OBu^t)-Gly-OH (XVI). To a suspension of compound XV (80 g, 0.308 mol) in DMF (2 liters) was added Et_3N (43.3 ml, 0.308 mol), followed by Z-Ala-ONSu (109.5 g, 0.342 mol). After 45 min stirring a clear solution was formed; then was added *N*-methylmorpholine (33 ml, 0.308 mol) and the reaction was allowed to proceed for 22 hr. After treatment with *N*-(2-aminoethyl)piperazine (13 ml) for 1 hr the solvent was removed and the residue distributed between EtOAc and 1% $KHSO_4$ solution; the organic phase was washed with H_2O, dried on Na_2SO_4, and evaporated to dryness. The solid was precipitated from EtOAc with diisopropylether and crystallized from MeOH–H_2O; yield: 119.5 g (83%); mp 117–118°C; $[\alpha]_D^{20}$ −33.1° and $[\alpha]_{546}^{20}$ −39.4° (c 2, MeOH); tlc: 27, 6.

Anal. Calcd. for $C_{22}H_{31}O_3N_8$ (465.51): C, 56.76; H, 6.71; N, 9.03. Found: C, 56.76; H, 6.88; N, 9.05.

H-Ala-Glu(OBu^t)-Gly-OH (XVII). Compound XVI (117.5 g, 0.252 mol) was hydrogenated in MeOH (1.5 liters) and H_2O (1 liter) as described for XV; yield: 83.3 g (98%); $[\alpha]_D^{20}$ −4.2° and $[\alpha]_{546}^{20}$ −5.1° (c 2, 80% AcOH); tlc: 2, 6, 23.

Anal. Calcd. for $C_{14}H_{25}N_3O_6 \cdot 1/4H_2O$ (335, 87): C, 50.06; H, 7.65; N, 12.51. Found: C, 50.28; H, 7.71; N, 12.48.

BOC-Tyr(Bu^t)-Ala-Glu(OBu^t)-Gly-OH (XVIII). The tripeptide derivative XVII (83.3 g, 0.248 mol) was coupled with BOC-Tyr(Bu^t)-ONSu (118.5 g, 0.272 mol) as described for XVI. The product was crystallized from EtOAc (2.5 liter)–THF (100 ml) and from 2-propanol; yield: 142 g (88%); mp 178–179°C; $[\alpha]_D^{20}$ −19.9° and $[\alpha]_{546}^{20}$ −16.8° (c 2, MeOH); tlc: 2, 6, 27.

Anal. Calcd. for $C_{32}H_{30}N_4O_{10}$ (650.78): C, 59.06; H, 7.74; N, 8.61. Found: C, 59.12; H, 7.69; N, 8.66.

BOC-Tyr(Bu^t)-Ala-Glu(OBu^t)-Gly-Thr(Bu^t)-Phe-OH (XIX). Pivaloyl chloride (0.58 ml; 4.75 mmol) was added at −10°C to a solution of the protected tetrapeptide XVIII (3.25 g, 5.0 mmol) and *N*-methylmorpholine (0.55 ml, 5.0 mmol) in DMF (70 ml). The mixture was stirred at −10°C for 15 min and then combined with a solution of H-Thr(Bu^t)-Phe-OH · $1/2H_2O$ (2.32 g, 7.0 mmol) in DMF (30 ml) and Et_3N (0.98 ml, 7.0 mmol) precooled to −10°C. After 2 hr stirring at 0°C and overnight at room temperature the bulk of the solvent was evaporated and the residue distributed between EtOAc and H_2O containing $KHSO_4$ (2 g). The organic phase was washed neutral with water. The product crystallized from the EtOAc solution on standing in the cold; yield: 3.0 g (63%); mp 172–174°C; $[\alpha]_D^{20}$ + 18.1° and $[\alpha]_{546}^{20}$ + 22.0° (c 1.1, DMF), tlc: 27, 2; amino acid ratios in acid hydrolysate: Tyr 1.00 (1) Ala 1.01 (1) Glu 1.02 (1) Gly 1.00 (1) Thr 0.99 (1) Phe 1.01 (1).

Anal. Calcd. for $C_{49}H_{74}N_6O_{13} \cdot H_2O$ (973.18): C, 60.46; H, 7.87; N, 8.64. Found: C, 60.67; H, 7.87; N, 8.65.

Boc-Tyr(But)-Ala-Glu(*OBu^t*)-*Gly-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-Glu(OBu^t)-Leu-Ser(Bu^t)-Arg(HBr)-Leu-Arg(HBr)-Asp(OBu^t)-Ser(Bu^t)-Ala-Arg(HBr)-Leu-Gln-Arg(HBr)-Leu-Leu-Gln-Gly-Leu-Val-NH$_2$* (*XX*). The protected hexapeptide XIX (0.312 g, 0.321 mmol) was coupled to H-Thr(Bu^t)-Ser(Bu^t)-Glu(OBu^t)-Leu-Ser(Bu^t)-Arg(HBr)-Leu-Arg(HBr)-Asp(OBu^t)-Ser(Bu^t)-Ala-Arg(HBr)-Leu-Gln-Arg(HBr)-Leu-Leu-Gln-Gly-Leu-Val-NH$_2$ · HBr (0.336 g, 0.107 mmol) and the product isolated as described for X; yield: 0.33 g; tlc: 2 (the product is contaminated by a trace impurity with lower R_f value); amino acid ratios of acid hydrolysate: Tyr 0.87 (1) Ala 1.98 (2) Glu 4.02 (4) Gly 1.99 (2) Thr 1.95 (2) Phe 0.91 (1) Ser 3.03 (3) Leu 5.90 (6) Arg 3.94 (4).

H-Tyr-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Asp-Ser-Ala-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH$_2$ (*GIP-SN; XXI*). The protected heptacosapeptide XX (0.32 g, 0.0796 mmol) was added to ice-cold anhydrous trifluoroacetic acid (11 ml) containing 10% anisole. The resulting solution was kept 150 min at room temperature, then Et_2O was added. The precipitate was collected, washed extensively with Et_2O, and dried over KOH pellets. The crude heptacosapeptide (0.28 g) was dissolved in 1% AcOH and applied to a SP-Sephadex C-25 column (54 × 2 cm) equilibrated with 0.05 *M* ammonium acetate buffer (pH 6.5). The column was developed with ca. 1700 ml 0.05 *M* (pH 7.1) followed by 0.075 *M* ammonium acetate buffer (pH 7.1) at a flow rate of 75 ml/hr; fractions of 75 ml each were collected after the buffer change and extinction at 206 nm was determined (for elution profile see Ref. (*1*)). Fractions 11–36 of the 0.075 *M* eluate were pooled and the product was isolated by lyophilization as described for XI; yield: 156 mg (52%) of fluffy material, which behaved homogeneously on silica gel tlc (solvent system 6; see Fig. 1) and on "isoelectric focusing" (Fig. 5); amino acid ratios in the acid hydrolysate: Tyr 1.01 (1) Ala 2.02 (2) Glu 3.95 (4) Gly 1.99 (2) Thr 1.96 (2) Phe 1.04 (1) Ser 2.98 (3) Leu 5.85 (6) Arg 4.04 (4) Asp 1.04 (1) Val 0.96 (1); peptide content: 80.2%.

Phe^1,Phe^2,Trp^3,Lys^4-Secretin

Z-Lys(Boc)-Thr(Bu^t)-Phe-OH (*XXII*). Z-Lys(Boc)-ONSu (14.7 g, 30.7 mmol) was reacted with H-Thr(Bu^t)-Phe-OH · 1/2H_2O (9.94 g, 29.2 mmol) in DMF (200 ml) containing Et_3N (4.1 ml, 29.2 mmol) and pyridine (2.35 ml, 29.2 mmol). After 12 hr the bulk of the solvent was evaporated and the residue distributed between EtOAc and 1% $KHSO_4$. The organic phase was washed twice more with 1% $KHSO_4$ and H_2O, dried over Na_2SO_4, and evaporated. The oil was reprecipitated twice from EtOAc with Et_2O–petroleum ether; yield: 17.4 g (87%); mp 98–101°C; $[\alpha]_D^{20}$ + 23.0° and $[\alpha]_{546}^{20}$ + 27.7° (c 1.1, MeOH); tlc: 36, 27.

Anal. Calcd. for $C_{36}H_{52}N_4O_9$ (684.84): C, 63.14; H, 7.65, N, 8.18. Found: C, 63.32; H, 7.69; N, 8.20.

H-Lys(Boc)-Thr(Bu^t)-Phe-OH (*XXIII*). Compound XXII (17 g, 24.82 mmol) was hydrogenated in 90% MeOH (500 ml) as described for XV. The product was

crystallized from MeOH–Et_2O; yield: 13.2 g (95%); mp 169–171°C; $[\alpha]_D^{20}$ + 66.6° and $[\alpha]_{546}^{20}$ + 79.9° (c 1, MeOH); tlc: 27, 36.

Anal. Calcd. for $C_{28}H_{46}N_4O_7$ (550.7): C, 61.07; H, 8.42; N, 10.17. Found: C, 60.94; H, 8.38; N, 10.12.

Z-Trp-Lys(Boc)-Thr(Bu^t)-Phe-OH (XXIV). Z-Trp-ONp (1.74 g, 3.8 mmol) was reacted with compound XXIII (2.24 g, 4.06 mmol) in DMF (60 ml) containing Et_3N (0.57 ml, 4.06 mmol) for 72 hr as described for XXII. The product was crystallized from EtOAc–Et_2O; yield: 2.9 g (88%); mp 158–160°C; $[\alpha]_D^{20}$ + 13.1° and $[\alpha]_{546}^{20}$ + 16.0° (c 1, MeOH); tlc: 2, 27.

Anal. Calcd. For $C_{47}H_{62}O_6N_{10}$ (871.05): C, 64.81; H, 7.17; N, 9.65. Found: C, 64.86; H, 7.20; N, 9.63.

H-Trp-Lys(Boc)-Thr(Bu^t)-Phe-OH (XXV). The protected tetrapeptide XXIV (2.53 g, 2.9 mmol) was hydrogenated in 90% MeOH (200 ml) for 48 hr as described for XV. The residue was crystallized from MeOH–H_2O; yield: 1.94 g (91%); mp 162–164°C; $[\alpha]_D^{20}$ + 47.2° and $[\alpha]_{546}^{20}$ + 57.3° (c 1, MeOH); tlc: 2, 27.

Anal. Calcd. for $C_{39}H_{56}N_6O_8$ (736.92): C, 63.57; H, 7.66; N, 11.40. Found: C, 63.26; H, 7.68; N, 11.59.

Z-Phe-Trp-Lys(Boc)-Thr(Bu^t)-Phe-OH (XXVI). Z-Phe-ONSu (1.23 g, 3.1 mmol) was coupled with the tetrapeptide derivative XXV (2.1 g, 2.8 mmol) in DMF (50 ml) containing Et_3N (0.39 ml, 2.8 mmol) and pyridine (0.226 ml, 2.8 mmol) for 48 hr as described for XXII. The product was precipitated from MeOH with Et_2O; yield: 2.5 g (87%); mp 163–165°C; $[\alpha]_D^{20}$ + 6.77° and $[\alpha]_{546}^{20}$ + 8.4 (c 1, 80% AcOH); tlc: 27, 28, 36.

Anal. Calcd. for $C_{56}H_{71}N_7O_{11}$ (1018.23): C, 66.06; H, 7.03; N, 9.63. Found: C, 65.66; H, 7.02; N, 9.51.

H-Phe-Trp-Lys(Boc)-Thr(Bu^t)-Phe-OH (XXVII). Compound XXVI (1.5 g, 1.47 mmol) was hydrogenated over Pd-black for 72 hr in MeOH–H_2O–1-BuOH (3 : 1 : 1 v/v; 200 ml); the filtrate was concentrated to small volume and the product obtained on addition EtOAc–Et_2O (1 : 1 v/v); yield: 1.0 g (79%); mp 193–194°C (dec.); $[\alpha]_D^{20}$ + 42.35° and $[\alpha]_{546}^{20}$ + 51.12° (c 1, AcOH); tlc: 2, 27, 36.

Anal. Calcd. for $C_{48}H_{65}N_7O_9$ (884.095): C, 65.21; H, 7.41; N, 11.09. Found: C, 65.30; H, 7.26; N, 11.05.

Boc-Phe-Phe-Trp-Lys(Boc)-Thr(Bu^t)-Phe-OH (XXVIII). Boc-Phe-ONSu (0.434 g, 1.2 mmol) was added to a solution of compound XXVII (0.95 g, 1.07 mmol) in DMF (40 ml) containing Et_3N (0.15 ml, 1.07 mmol) and pyridine (9.09 ml, 1.1 mmol). The mixture was stirred for 12 hr and then acidified with $KHSO_4$ (0.41 g) in H_2O (20 ml). The product obtained on addition of H_2O was reprecipitated from MeOH–Et_2O–petroleum ether; yield: 0.95 g (79%); mp 166–168°C; $[\alpha]_D^{20}$ + 7.4° and $[\alpha]_{546}^{20}$ + 9.2° (c 1, 80% AcOH); tlc: 2, 27, 36. Amino acid ratios in acid hydrolysate: Phe 2.87 (3) Trp 0.9 (1) Lys 1.01 (1) Thr 0.99 (1).

Anal. Calcd. for $C_{62}H_{82}N_8O_{12}$ (1131.39): C, 65.82; H, 7.31; N, 9.90 Found: C, 65.55; H, 7.27; N, 9.74.

Boc-Phe-Phe-Trp-Lys(Boc)-Thr(*Bu^t*)-*Phe-Thr(Bu^t)-Ser(Bu^t)-Glu(OBu^t)-Leu-Ser(Bu^t)-Arg(HBr)-Leu-Arg(HBr)-Asp(OBu^t)-Ser(Bu^t)-Ala-Arg(HBr)-Leu-Gln-Arg(HBr)-Leu-Leu-Gln-Gly-Leu-Val-NH$_2$ (XXIX).* The protected hexapeptide XXVIII (0.363 g, 0.321 mmol) was coupled to H-Thr(Bu^t)-Ser(Bu^t)-Glu(OBu^t)-

Leu-Ser(Bu^t)-Arg(HBr)-Leu-Arg(HBr)-Asp(OBu^t)-Ser(Bu^t)-Ala-Arg(HBr)-Leu-Gln-Arg(HBr)-Leu-Leu-Gln-Gly-Leu-Val-NH_2 · HBr (0.336 g, 0.107 mmol) and the product isolated as described for X; yield: 0.36 g; tlc: 2 (practically homogeneous); amino acid ratios in the acid hydrolysate: Phe 2.82 (3) Lys 1.07 (1) Thr 1.92 (2) Ser 2.86 (3) Glu 2.99 (3) Leu 5.71 (6) Arg 4.40 (4) Asp 1.09 (1) Ala 1.00 (1) Gly 1.03 (1) Val 0.95 (1).

H-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Asp-Ser-Ala Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH_2 (SOMA-SN; XXX). The protected heptacosapeptide XXIX (0.32, 0.077 mmol) was dissolved in ice-cold anhydrous trifluoroacetic acid (11.5 ml) containing 10% anisole and ethanedithiol (0.5 ml). The solution was kept under argon for 150 min at room temperature and the precipitate obtained on addition of Et_2O was collected, washed extensively with Et_2O, and dried over KOH pellets. The crude product (0.285 mg) was dissolved in 5% AcOH (20 ml) and applied to a Servacel CM-23 column (20 × 3 cm) equilibrated with 0.35 *M* ammonium acetate buffer (pH 7.25) and eluted successively with ca. 4000 ml 0.35 *M* (pH 7.25) and 0.5 *M* ammonium acetate buffer (pH 7.25) at a flow rate of 75 ml/hr; fractions of 75 ml each were collected after the buffer change. The peptide distribution was monitored by continuous spectrophotometric measurements at 206 and 248 nm of the eluates. Fractions 16–50 of the 0.5 *M* eluate were pooled and the desired product was isolated by lyophilization as described for XI; yield: 139 mg (45%) of fluffy material, which appeared homogeneous on silica gel tlc (solvents system 6; Fig. 1) and on "isoelectric focusing" (Fig. 5); amino acid ratios in the acid hydrolysate: Phe 2.92 (3) Trp 0.95 (1) Lys 0.99 (1) Thr 1.95 (2) Ser 2.94 (3) Glu 3.01 (3) Leu 6.09 (6) Arg 4.02 (4) Asp 0.99 (1) Ala 1.02 (1) Gly 1.03 (1) Val 0.97 (1); peptide content: 81.3%.

N$^{\alpha}$-3-(4-Hydroxyphenyl)propionyl-β-alanyl-secretin

3-(4-Hydroxyphenyl)propionic acid 4-nitrobenzyl ester (XXXI). To a solution of 3-(4-hydroxyphenyl)propionic acid (1.66 g, 10 mmol) in DMF (80 ml) 4-nitrobenzyl bromide (2.16 g, 10 mmol) and Et_3N (1.4 ml, 10 mmol) were added. The mixture was stirred for 12 hr and then evaporated. The residue was distributed between EtOAc and H_2O and the organic phase was extracted with 10% $KHCO_3$ and H_2O, dried over Na_2SO_4, and evaporated to dryness. The residue was crystallized from diisopropylether–petroleum ether; yield: 1.6 g (53%); mp 80–82°C; tlc: 36.

Anal. Calcd. for $C_{16}H_{15}NO_5$ (301.31): C, 63.7; H, 5.02; N, 4.65. Found: C, 63.93; H, 5.07; N, 4.72.

3-(4-tert-Butoxyphenyl)propionic acid 4-nitrobenzyl ester (XXXII). Compound XXXI (4.5 g, 15 mmol) in CH_2Cl_2 (200 ml) was reacted at 0°C with liquid isobutene (100 ml) and H_2SO_4 (0.5 ml). The reaction mixture was kept in a closed vessel for 72 hr at room temperature and then poured into 0.4 *N* $KHCO_3$ (100 ml). The bulk of the organic solvent was evaporated and the aqueous phase extracted several times with Et_2O. The combined extracts were washed with 0.5 *N* NaOH and H_2O, dried (Na_2SO_4), and evaporated. The solid residue was taken up in petroleum

ether, insoluble material was removed by filtration, and the filtrate evaporated to dryness; yield: 4.5 g (83%) of crystalline product; mp 30–32°C; tlc: 36.

Anal. Calcd. for $C_{20}H_{23}NO_5$ (357.42): C, 67.15; H, 6.48; N, 3.92. Found: C, 67.23; H, 6.38; N, 3.82.

3-(4-tert-Butoxyphenyl)propionic acid (XXXIII). Compound XXXII (2.86 g, 8.0 mmol) was saponified in dioxane (500 ml) with 2 *N* NaOH (8 ml) in 2 hr at 70°C and 12 hr at room temperature. After acidification with 1 *N* HCl (16 ml), the bulk of the solvent was removed and the residue distributed between Et_2O and 0.5 *N* $KHCO_3$ (40 ml). The aqueous phase was acidified with 1 *N* HCl and extracted several times with EtOAc. The combined extracts were washed with H_2O, dried (Na_2SO_4), and evaporated to dryness. The residue was taken up in *n*-heptane, insoluble material was filtered off, and the filtrate evaporated to dryness; yield: 1.3 g (73%) of crystalline product; mp 29–31°C; tlc: 36.

Anal. Calcd. for $C_{13}H_{18}O_3$ (222.28): C, 70.20; H, 8.17. Found: C, 70.01; H, 8.40.

N-Hydroxysuccinimido 3-(4-tert-butoxyphenyl)propionate (XXXIV). To a solution of compound XXXIII (1.11 g, 5.0 mmol) and HONSu (0.58 g, 5.0 mmol) in EtOAc (50 ml) DCC (1.03 g, 5.0 mmol) was added at 0°C. The mixture was stirred at 0°C for 12 hr, filtered, and the filtrate was evaporated. The residue was crystallized from 2-propanol; yield 1.3 g (81%); mp 115–116°C; tlc: 27, 36.

Anal. Calcd. for $C_{17}H_{21}N_1O_5$ (319.36): C, 63.90; H, 6.63; N, 4.38. Found: C, 63.01; H, 6.71; N, 4.26.

3-(4-tert-Butoxyphenyl)propionyl-βAla-OH (XXXV). Compound XXXIV (4.45 g, 14.0 mmol) was added at 0°C to a solution of H-βAla-OH (2.48 g, 28.0 mmol) in DMF–H_2O (9 : 1 v/v; 100 ml) containing Et_3N (3.9 ml, 28.0 mmol). The mixture was stirred 2 hr at 0°C and 12 hr at room temperature and worked up as described for XXII. The product was crystallized from diisopropylether–petroleum ether; yield: 3.4 g (83%); mp 70–72°C; tlc: 27, 36.

Anal. Calcd. for $C_{16}H_{23}NO_4$ (293.37): C, 65.50; H, 7.89; N, 4.77. Found: C, 65.44; H, 7.98; N, 4.80.

N-Hydroxysuccinimido 3-(4-tert-butoxyphenyl)propionyl-βAla-ONSu (XXXVI). To a solution of compound XXXV (3.5 g, 11.9 mmol) and HONSu (1.38 g, 11.9 mmol) in DMF (60 ml) DCC (2.47 g, 11.9 mmol) was added at 0°C. The mixture was stirred at 0°C for 12 hr, filtered, and the filtrate evaporated. The product was crystallized twice from 2-propanol; yield: 2.9 g (63%); mp 106–107°C.

Anal. Calcd. for $C_{20}H_{26}N_2O_6$ (390.44): C, 61.50; H, 6.71; N, 7.18. Found: C, 61.76; H, 6.96; N, 7.27.

Z-His-Ser(Bu^t)-Asp(OBu^t)-Gly-Thr(Bu^t)-Phe-OH (XXXVII). *tert*-Butylnitrite (1.02 ml, 8.6 mmol) was added at −15°C to a solution of *Z*-His-$NHNH_2$[10] (2.37 g, 7.8 mmol) in DMF (60 ml) containing 5.9 *N* HCl in dioxane (6.0 ml, 31.2 mmol). The mixture was stirred at −15°C for 10 min, then it was cooled to −50°C and Et_3N (4.27 ml, 31.2 mmol) was added, followed by the chilled solution of H-Ser(Bu^t)-Asp(OBu^t)-Gly-Thr(Bu^t)-Phe-OH · H_2O (4.75 g, 6.5 mmol) in DMF (80 ml) containing *N*-methylmorpholine (0.715 ml, 6.5 mmol). After 12 hr stirring at

[10] Crystallized from MeOH: mp 164–166°C; $[\alpha]_D^{20}$ − 6.3° and $[\alpha]_{546}^{20}$ − 7.8° (c 1, DMF); Lit. (22): mp 171–173°C.

4°C and an additional 8 hr at room temperature the bulk of the solvent was evaporated and the residue distributed between $CHCl_3$–1-BuOH (9 : 1 v/v; 200 ml) and 1% $KHSO_4$ (130 ml). The organic phase was washed with H_2O, concentrated to small volume and poured into EtOAc. The precipitate was collected and reprecipitated from MeOH with EtOAc; yield: 5.1 g (81%); mp 185–189°C; $[\alpha]_D^{20}$ – 3.7° and $[\alpha]_{546}^{20}$ – 4.7° (c 1, MeOH); tlc: 2.

Anal. Calcd. for $C_{48}H_{68}N_8O_{13} \cdot H_2O$ (983.15): C, 58.64; H, 7.18; N, 11.40. Found: C, 58.75; H, 7.12; N, 11.25.

H-His-Ser(Bu^t)-Asp(OBu^t)-Gly-Thr(Bu^t)-Phe-OH · AcOH (*XXXVIII*). The protected hexapeptide XXXVII (4.3 g, 4.37 mmol) was hydrogenated over Pd-black in 90% MeOH (150 ml) containing AcOH (1 ml) for 48 hr. The catalysat was filtered off and the filtrate evaporated. The residue was reprecipitated from MeOH with EtOAc–Et_2O (1 : 1 v/v); yield: 3.2 g (82.5%); mp 155–158°C; $[\alpha]_D^{20}$ + 21.4° and $[\alpha]_{546}^{20}$ + 25.3° (c 1, MeOH); tlc: 2, 6.

Anal. Calcd. for $C_{40}H_{62}N_8O_{11} \cdot AcOH$ (891.04): C, 56.62; H, 7.47; N, 12.57. Found: C, 56.62; H, 7.51; N, 13.01.

3-(4-tert-Butoxyphenyl)propionyl-βAla-His-Ser(Bu^t)-Asp(OBu^t)-Gly-Thr-(Bu^t)-Phe-OH (*XXXIX*). To a solution of the hexapeptide derivative XXXVIII (1.0 g, 1.12 mmol) in DMF (25 ml) containing Et_3N (0.315 ml, 2.24 mmol) *N*-hydroxy-succinimido 3-(4-*tert*-butoxyphenyl)propionyl-βAla-ONSu (0.585 g, 1.5 mmol) was added. After 12 hr stirring the bulk of the solvent was evaporated and the residue distributed between $CHCl_3$ and H_2O containing $KHSO_4$ (0.3 g). The organic phase was washed with H_2O and evaporated. The residue was suspended for 1 hr in 1-BuOH–EtOAc and then collected; yield: 1.2 g (98%); mp 117–120°C; $[\alpha]_D^{20}$ + 5.5° and $[\alpha]_{546}^{20}$ + 6.9 (c 1, DMF); tlc: 2, 6.

Anal. Calcd. for $C_{56}H_{83}N_9O_{14}$ (1106.34): C, 60.78; H, 7.56; N, 11.39. Found: C, 60.48; H, 7.65; N, 11.39.

3-(4-tert-Butoxyphenyl)propionyl-βAla-His(Adoc)-Ser(Bu^t)-Asp(OBu^t)-Gly-Thr(Bu^t)-Phe-OH (*XL*). Compound XXXIX (1.0 g, 0.9 mmol) in DMF (35 ml) containing Et_3N (0.38 ml, 2.7 mmol) was reacted at 0°C with Adoc-F (0.36 g, 1.8 mmol). The mixture was stirred overnight at 4°C; then the solvent was evaporated and the residue distributed between $CHCl_3$ and 1% $KHSO_4$ (50 ml); the organic layer was washed with H_2O, dried (Na_2SO_4), and concentrated. The product was obtained on addition of Et_2O; yield: 1.06 g (92%); mp 144–145°C; $[\alpha]_D^{20}$ + 17.8° and $[\alpha]_{546}^{20}$ + 21.3° (c 1.03, MeOH); tlc: 2, 27; amino acid ratios in acid hydrolysate: βAla 0.91 (1) His 0.99 (1) Ser 1.01 (1) Asp 0.97 (1) Gly 1.01 (1) Thr 0.99 (1) Phe 1.00 (1).

Anal. Calcd. for $C_{67}H_{97}N_9O_{16}$ (1284.57): C, 62.65; H, 7.61; N, 9.81. Found: C, 62.50; H, 7.82; N, 9.67.

3-(4-tert-Butoxyphenyl)propionyl-βAla-His(Adoc)-Ser(Bu^t)-Asp(OBu^t)-Gly-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-Glu(OBu^t)-Leu-Ser(Bu^t)-Arg(HBr)-Leu-Arg(HBr)-Asp(OBu^t)-Ser(Bu^t)-Ala-Arg(HBr)-Leu-Gln-Arg(HBr)-Leu-Leu-Gln-Gly-Leu-Val-NH$_2$ (*XLI*). Compound XL (0.683, 0.53 mmol) was added to a solution of H-Thr(Bu^t)-Ser(Bu^t)-Glu(OBu^t)-Leu-Ser(Bu^t)-Arg(HBr)-Leu-Arg(HBr)-Asp(OBu^t)-Ser(Bu^t)-Ala-Arg(HBr)-Leu-Gln-Arg(HBr)-Leu-Leu-Gln-Gly-Leu-Val-NH_2 · HBr (0.55 g, 0.175 mmol) in DMF (8 ml), DMA (24 ml), and

HMPA (12 ml) containing Et_3N (24.5 μl, 0.175 mmol) and HOBt (92 mg, 0.683 mmol), followed at −15°C by DCC (0.11 g, 0.53 mmol). The mixture was stirred 48 hr at 4°C and 24 hr at room temperature; then additional protected hexapeptide XL (0.225 g, 0.175 mmol) and HOBt (30 mg, 0.203 mmol) was added, followed at −15°C by DCC (36 mg, 0.175 mmol). The reaction was allowed to proceed for 72 hr at room temperature; then the mixture was filtered into warm EtOAc (400 ml). The precipitate was collected, washed with EtOAc, suspended in H_2O–MeOH (3:1 v/v; 100 ml), and the resulting gel was concentrated after addition of 1-pentanol (10 ml) to ca. 20 ml. On addition of EtOAc the product was collected by filtration; yield 0.58 g; tlc: 2 (the product is slightly contaminated by two impurities with lower R_f values); amino acid analysis in the acid hydrolysate: βAla 1.07 (1) His 1.01 (1) Ser 3.98 (4) Asp 2.00 (2) Gly 1.99 (2) Thr 1.97 (2) Phe 1.02 (1) Glu 3.08 (3) Leu 5.84 (6) Arg 4.01 (4) Ala 1.04 (1) Val 0.96 (1).

3-(4-Hydroxyphenyl)propionyl-βAla-His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Asp-Ser-Ala-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH_2 (DATA-SN; XLII). The protected heptacosapeptide derivative XLI (0.48 g, 0.11 mmol) was dissolved in ice-cold anhydrous trifluoroacetic acid containing 10% anisole (15 ml). The solution was kept under argon at room temperature for 150 min and then evaporated. The residue was lyophilized from H_2O; yield 0.43 g of crude product (XLII-A); peptide content: 72%. The crude product XLII-A was purified by two different procedures.

(a) The product XLII-A (150 mg) was dissolved in 1% AcOH (5 ml) and applied to a SP-Sephadex G-25 column (30 × 1 cm) previously equilibrated with 0.02 *M* ammonium acetate buffer (pH 6.0). The column was developed at a flow rate of 23.5 ml/hr successively with the following ammonium acetate buffers: 350 ml of 0.02 *M* (pH 6.0), 600 ml of 0.05 *M* (pH 6.0) and 0.2 *M* buffer (pH 6.6); fractions of 25 ml each were collected and absorbancy at 206 and 254 nm was determined.

Fractions 2–32 of the 0.2 *M* eluate were pooled and lyophilized as described for XI; yield: 93 mg (peptide content: 82%) of a product (XLII-B1), which appeared contaminated by small amounts of impurities on silica gel tlc (solvent system 6; see Fig. 1). The final purification was achieved by two methods.

(i) Partition chromatography using the solvent system 2-BuOH–0.05 *M* AcOH–EtOH (1:1:0.05 v/v; pH 3.4). A Sephadex G-25 F column (200 × 1 cm) was previously equilibrated with the lower phase of the solvent system and then eluted with 150 ml of the upper phase. Then the compound XVII-B_1 (30 mg) dissolved in the upper phase (2 ml) was applied and eluted with this upper phase at a flow rate of 13 ml/hr; individual fractions of 3.25 ml each were collected and absorbancy at 206 nm was measured;[11] the desired pure material was detected by tlc in solvent system 6 in the tubes No 33–39; these were pooled and evaporated. The product was isolated by lyophilization as described for XI; from fractions 28–32 and 40–44 additional pure product was obtained by rechromatography; yield: 20 mg (42.4%). The product XLII-C_1 was shown to be homogeneous on silica gel tlc (solvent

[11] Turbidity of eluant during passing the measuring cell was avoided by continuous admixture of 10% (v/v) ethanol.

system 6; Fig. 1), "isoelectric focusing" (Fig. 5), and free-flow electrophoresis (for experimental conditions see under (ii)); amino acid analysis in the acid hydrolysate: βAla 1.07 (1) His 1.01 (1) Ser 3.98 (4) Asp 2.00 (2) Gly 1.99 (2) Thr 1.97 (2) Phe 1.02 (1) Glu 3.08 (3) Leu 5.90 (6) Arg 4.01 (4) Ala 1.04 (1) Val 0.96 (1); peptide content: 85.3%; 3-(4-hydroxyphenyl)propionic acid: 0.95 (1); determined spectrophotometrically using the extinction coefficients $\epsilon_{275.5} = 1.52.10^3$ (in H_2O) and $\epsilon_{294} = 2.44.10^3$ (in 0.1 *N* NaOH) obtained for 3-(4-hydroxyphenyl)propionic acid (Fluka AG; purum).

(ii) Free-flow electrophoresis using a VaP_2 apparatus and 0.022 *M* ammonium acetate buffer (pH 6.9) as electrolyte. A constant flow rate of 120 ml/hr for the electrolyte solution and a field strength of 48 V/cm was applied. Compound XLII-B_1 (20 mg) was dissolved in 0.022 *M* ammonium acetate buffer (pH 6.9) and injected continuously over a period of 3 hr into the separation chamber above the position of collecting tube No. 30. A constant flow of electrolyte was maintained for an additional 3 hr and a volume of 75 ml electrolyte per tube was collected. The peptide distribution (Fig. 2) was determined by measuring the absorbancy at 220 nm. Fractions 32–43 were pooled and lyophilized as described for XI; from fractions 44–49 additional pure material was obtained after reelectrophoreses; yield: 14 mg (42.7%). The product XLII-D_1 was shown to be homogeneous on silica gel tlc (solvent system 6; Fig. 1) and "isoelectric focusing"; amino acid analysis in the acid hydrolysate: βAla 1.09 (1) His 0.98 (1) Ser 4.06 (4) Asp 2.03 (2) Gly 2.02 (2) Thr 2.00 (2) Phe 1.04 (1) Glu 3.06 (3) Leu 5.96 (6) Arg 4.04 (4) Ala 1.06 (1) Val 0.96 (1); peptide content: 82.0%; 3-(4-hydroxyphenyl)propionic acid: 0.93 (1) (determined spectrophotometrically as described for XLII-C_1).

(b) A Sephadex G-25 F column (180 × 4 cm) previously equilibrated with the lower phase of the 2-BuOH–0.025 *M* AcOH–EtOH (1 : 1 : 0.05 v/v; pH 4.5) system was eluted with the void volume of the upper phase of latter solvent system. Then crude heptacosapeptide derivative XLII-A (320 mg; peptide content: 71%) dissolved in 30 ml of upper phase was applied to the column and eluted with the same solvent mixture at a flow rate of 80 ml/hr; fractions of 40 ml each were collected and the absorbancy at 206 nm was determined.[11] Fractions 26–31 were combined and the bulk of the organic solvents was evaporated; the aqueous solution was lyophilized; yield: 228 mg. This was dissolved in 1% AcOH (30 ml) and applied to a Servacel CM-23 (30 × 3 cm) column equilibrated with 0.02 *M* ammonium acetate buffer (pH 5.8). The column was developed at a flow rate of 160 ml/hr with 4160 ml 0.02 *M* (pH 5.8) buffer, followed by 0.05 *M* buffer (pH 6.0); fractions of 80 ml were collected and the absorbancy at 206 nm was determined (for elution profile see Fig. 4). Fractions 17–40 of the 0.05 *M* eluate were combined and the desired product was obtained by lyophilization as described for XI; yield: 149.5 mg (52%) of fluffy material, which was shown to be identical to above reported products XLII-C_1 and XLII-D_1 on tlc, "isoelectric focusing," and free-flow electrophoresis; amino acid analysis in the acid hydrolysate: βAla 0.85 (1) His 0.97 (1) Ser 4.13 (4) Asp 2.05 (2) Gly 2.03 (2) Thr 2.04 (2) Phe 1.01 (1) Glu 3.03 (3) Leu 5.95 (6) Arg 4.06 (4) Ala 0.99 (1) Val 0.96 (1); peptide content: 79.4%; 3-(4-hydroxyphenyl)propionic acid 0.96 (1) (determined spectrophotometrically as described for XLII-C_1).

RESULTS AND DISCUSSION

As reported previously (*1*), the hybrid hormones VIP-SN, GLU-SN, and GIP-SN were assayed for their potency in stimulating the adenylate cyclase in pancreatic plasma membrane preparations according to the method of Milutinovič *et al.* (*23*) and compared in terms of half-maximal and maximal stimulation of the enzyme with synthetic secretin (*24*) and natural VIP and glucagon preparations. The concentrations of the analogs and of VIP producing half-maximal stimulation of the enzyme ranged from 7.8×10^{-8} *M* (VIP-SN), over 3.1×10^{-7} *M* (GIP-SN), and 2.0 10^{-7} *M* (VIP) to 1.3×10^{-7} *M* (GLU-SN). These K_m values were higher than that of secretin (3.1×10^{-8} *M*). Compared to the maximal activation of the adenylate cyclase induced by secretin (100%), the analogs showed stimulations of 90% (VIP-SN), 50% (GIP-SN), and 30% (GLU-SN). The natural VIP preparation caused a stimulation of 60%, and the glucagon preparation gave no response.

On the contrary, the juice flow rate in isolated perfused cat pancreas induced by secretin, VIP, and the three hybrid hormones, reached practically identical maxima, albeit the potencies of VIP-SN, VIP, GIP-SN, and GLU-SN were 1.5-, 5-, 25-, and 90-fold weaker than that of secretin. Similarly, according to Gardner *et al.* (*25*), the high affinity secretin receptors in the dispersed pancreatic acinar cells allowed clear differentiation among the Tyr^{10}-secretin analogs, synthetized by Beyermann *et al.* (*8*). In terms of the apparent receptor affinity, the following order was determined: Tyr^{10}- > Ala^4, Tyr^{10}- > Glu^3, Tyr^{10}- = Gln^3, Tyr^{10}-secretin. The high affinity VIP receptor, however, recognized only Tyr^{10}-secretin at the concentrations used, thus prompting Gardner *et al.* to conclude: "substituting in secretin alanine for glycine in position 4 and/or tyrosine for leucine in position 10 makes the peptide more VIP-like in terms of structure, but not in terms of function." On the other hand, Bodanszky observed during his studies on the secretin-related peptides Gln^9-, Asn^{15}-, and Gln^9, Asn^{15}-$secretin^{5-27}$ that "the elimination of the (potential) negative charges at positions 9 and 15 seems to result in compounds that are less secretin-like and more similar to VIP in their activities" (*10*).

Our findings with the VIP-like hybrid analog VIP-SN are more in the line of Bodanszky's observation. In fact, VIP-SN possesses a 2-fold enhanced affinity to VIP receptors in liver plasma membrane preparations over the parent hormone secretin (Fig. 6). Its potency to inhibit ^{125}I-VIP binding in this assay system is 50-fold lower than that of VIP itself. SOMA-SN, GIP-SN, and GLU-SN are far less potent: their respective VIP receptor affinities are 2 10^4-, 4×10^4; and 5×10^4-fold lower than that of VIP. As recently determined (*26*), VIP-SN exhibits also in isolated intestinal epithelial cells a higher affinity for VIP receptors than secretin, whereas SOMA-SN, GLU-SN, and GIP-SN again poorly inhibit ^{125}I-VIP binding. In addition, for VIP-SN a marked potency to stimulate cyclic AMP accumulation in epithelial cells was observed. SOMA-SN, GLU-SN, and GIP-SN failed to produce any stimulation at the concentrations used (*27*).

Thus, the replacement of the hydrophobic dipeptide sequence glycyl-threonine (position 4–5 of the secretin molecule) by the hydrophobic one alanyl-valine (in

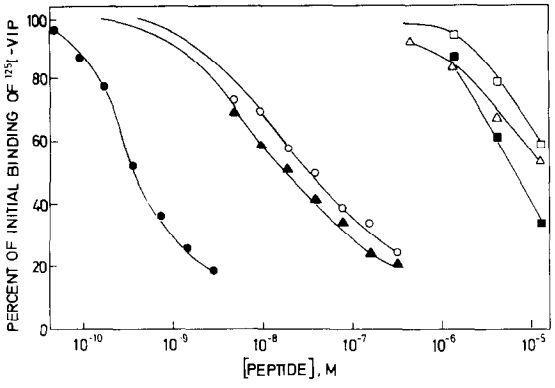


FIG. 6. Inhibition of ^{125}I-VIP binding to rat liver plasma membranes by unlabeled VIP (●), VIP-SN (▲), secretin (○), SOMA-SN (■), GIP-SN (△), and GLU-SN (□). Conditions of the binding assay were as described by M. Laburthe (*34*). Results are expressed as the percentage of radioactivity, specifically bound in the absence of added unlabeled peptide.

VIP-SN) reduces slightly (approximately a factor of two) the secretin character and concomitantly enhances the VIP character of the resulting analog by the same order of magnitude. Thus the structural requirements needed by the receptors for a clear differentiation between VIP and secretin are not related to the N-terminal region of these hormone sequences. This N-terminal part of the molecule bears sufficient sequence-dependent information to differentiate secretin, and possibly VIP, from the other two members of the glucagon family, i.e., glucagon and GIP. Elimination of the negatively charged carboxylate function of the aspartic acid residue in position 3 (GLU-SN) or change of its exact geography (GIP-SN) is accompanied by a remarkable loss of both secretin and VIP-receptor affinity. Therefore, the sequence portion 5–27 should contain the differentiating factors for VIP and secretin as well as the receptor recognition sites, which are amplified by three orders of magnitude through the additional binding sites of the sequence part 1–4 (Asp^3 and, possibly, the free α-amino function).

It is known from earlier studies (*10, 28, 29*) that $secretin^{5-27}$ is recognized by secretin-receptors and by VIP receptors, even if binding is weak. In order to investigate possible additive secretin-like information stored in the somatostatin sequence 6–13, the hybrid analog SOMA-SN was assayed for its adenylate cyclase stimulation potency in pancreatic plasma membrane preparations and was compared with the parent hormones secretin and somatostatin (Fig. 7). Cyclic and linear somatostatin were found to be unable to stimulate cyclic AMP production, even at concentrations up to $10^{-4}M$. Somatostatin exhibited no detectable secretin inhibitory activity if it was added up to the final concentration of 10^{-4} M to secretin. Our test system revealed no secretin agonist and antagonist character for somatostatin. On the other hand, SOMA-SN produces only a negligible increase in cyclic AMP production, although it strongly inhibits the secretin activity.[12] The

[12] For example: the addition of 5×10^{-8} M SOMA-SN to 5×10^{-8} M secretin resulted in a 30% inhibition of the secretin activity.

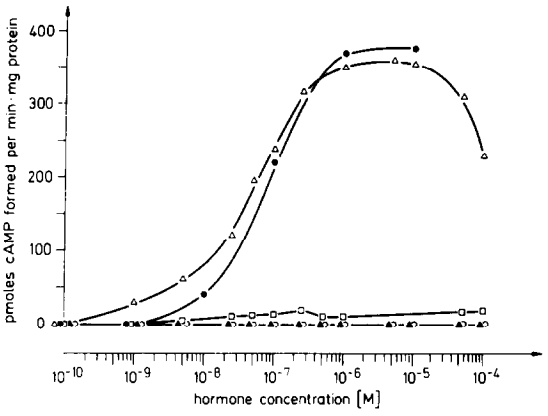


FIG. 7. Pancreatic adenylate cyclase activity for secretin (△), secretin $+10^{-6}$ cyclic somatostatin (●), SOMA-SN (□), and cyclic (○) and linear (▲) somatostatin. Assay conditions were as described in Ref. (*23*).

Lineweaver–Burk plot of the experimental data showed SOMA-SN to be a competitive inhibitor of secretin with markedly enhanced potency relative to that of secretin$^{5-27}$ (*10*). Therefore, strong additive binding sites must be located in the somatostatin moiety of SOMA-SN. This analog was also found to inhibit secretin-mediated cyclic AMP accumulation in neuroblastoma × glioma hybrid cells (*30*).

With the last analog in our series, DATA-SN, we attempted to gain knowledge about the role of the free α-amino function in the secretin molecule. Its dose–response curve related to the cyclic AMP production in pancreatic plasma membrane preparations is shown in Fig. 8, which indicates an apparent receptor

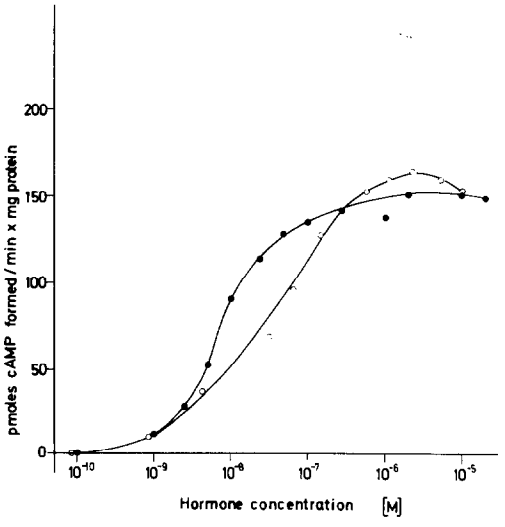


FIG. 8. Pancreatic adenylate cyclase activity for secretin (●) and DATA-SN (○). Assay conditions were as described in Ref. (*23*).

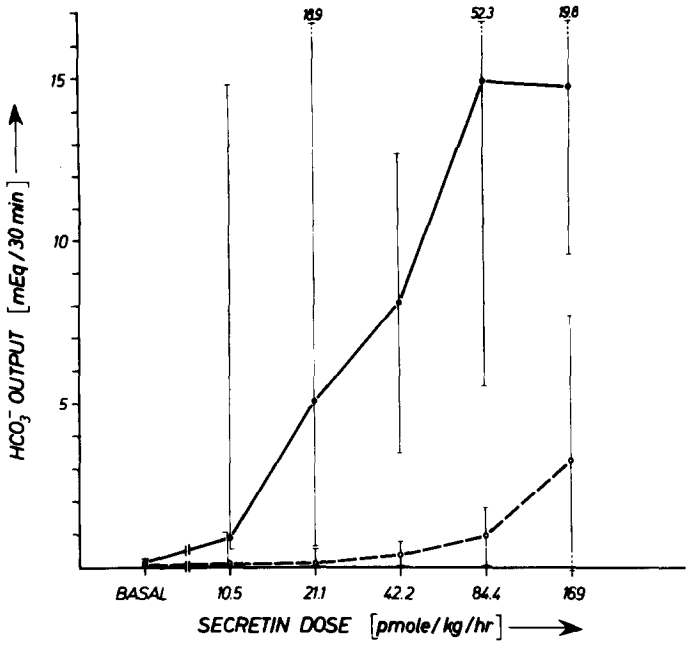


FIG. 9. Dose–response curves related to bicarbonate output induced by secretin (●——●) and DATA-SN (○——○). After continuous intravenous infusion for 1 hr, the doses were successively doubled, whereby the maximal dose was 915 ng = 169 pmol/kg/hr for secretin and 552 ng = 169 pmol/kg/hr for DATA-SN. The duodenal aspirates of seven healthy males were collected in the second half-hour of each infusion as described in Ref. (*35*), and used to calculate the statistical output (*36*). The given values are mean values ±SD (vertical bars).

affinity of 20% compared with that of secretin (100%). On the other hand, the maximal stimulation of the adenylate cyclase was found to be similar to that of secretin. In the *in vivo* assay, based on bicarbonate output in man, the analog appeared to be 5- to 10-fold less potent than secretin (Fig. 9).

The above results indicate a possible contribution of the free α-amino function of secretin to the formation of the optimally efficient hormone–receptor complex. On the basis of preliminary circular dichroism measurements, however, an interaction between the newly incorporated phenyl group and the rest of the molecule, and thus an indirect effect on the receptor affinity of this peptide, cannot be ruled out.

Similar effects could also result from the modifications within the other secretin analogs. Therefore their tendency to fold into ordered structures, possibly present in the hormone–receptor complex, were investigated in aqueous organic media, in order to better simulate more hydrophobic environments of the hormone bound to the receptor. No striking effects were observed.[13] On the other hand, insofar as the immunoreactivity can monitor structure-dependent conformational changes, the secretin analogs were tested for their ability to compete with ^{125}I-Tyr^6-secretin

[13] The results of these conformational studies will be reported elsewhere (*31*).

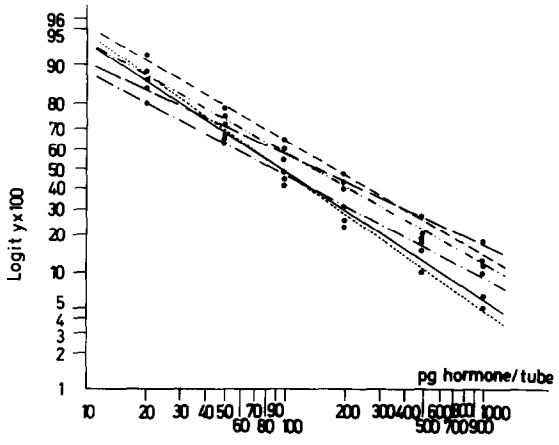


FIG. 10. Radioimmunoassays for secretin analogs using rabbit antisecretin antiserum AS 5013 and ^{125}I-Try^{6}-secretin according to Raptis *et al.* (*37*). The horizontal axis represents the log doses of added hormone and the vertical axis the logit of bound traces versus $B_0(29\%) \times 100$; B_0 = value of B with no unlabeled ligand in the system. Natural secretin (Mutt) (---); synthetic secretin (-·-·-); VIP-SN (···); GIP-SN (——); GLU-SN (–·–) and SOMA-SN (–··–). $B/B_0 = y$; Logit $y = \log y/1 - y$.

for secretin antibodies[14]. All the analogs exhibit very similar immunoreactivity, as shown in Fig. 10 and as reported previously for DATA-SN (*4*, *32*). The modifications in the N-terminal region do not significantly influence directly or indirectly the major secretin antigenic determinant(s), known to be located in the sequence portion 5–27 (*9*, *33*).

In conclusion, the data collected with the hybrid hormones demonstrate that, by analogy with other peptide hormones (*38*), secretin and possibly also the other members of the glucagon family bear in their primary structures distinct areas of information imprinted by the evolutionary process for the specific recognition by the receptor and efficient transduction of the hormonal message. Minimal structural changes in the N-terminal portion of the secretin molecule reduce its ability to fit optimally the receptor and concomitantly, to express the hormonal message with maximal efficiency, even if the invariant phenylalanine residue in position 6–probably indispensable, because of its bulky aromatic side chain, as a key binding site within the glucagon family–is retained in all analogs examined.

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[14] Immunized in rabbits against naturally occurring secretin (*39*).

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