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# Alternative Synthesis of Porcine Secretin and Apparent Autolysis of the Product[1)]

SHINYA KIYAMA,*,[a] KOUKI KITAGAWA,[a] TADASHI AKITA,[a]
WILLIAM Y. CHEY,[b] AVIDIN AYALP,[c] AKIKO OTSUKI,[d]
SUSUMU FUNAKOSHI,[d] NOBUTAKA FUJII,[d]
and HARUAKI YAJIMA[d]

*Faculty of Pharmaceutical Sciences, Tokushima University,[a] Sho-machi, Tokushima 770, Japan, The Genesee Hospital,[b] Rochester, N.Y. 14607, U.S.A., Faculty of Pharmacy, Ankara University,[c] Ankara, Turkey, and Faculty of Pharmaceutical Sciences, Kyoto University,[d] Sakyo-ku, Kyoto 606, Japan*

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Porcine secretin was synthesized by the trifluoromethanesulfonic acid deprotecting procedure. Release of the biologically important $His^1$-residue from synthetic secretin was noted when the secretin was incubated in an aqueous solution at 37 °C under nearly neutral conditions. Apparent autolysis of the N-terminal tetrapeptide, His–Ser–Asp–Gly, was examined by using 5 peptide analogs, and the participation of the $\beta$-carboxyl group of Asp and the basic amino acid, His, in this unusual phenomenon was deduced. Hydrolysis of the peptide bonds between Asp–Ser (15–16) and Asp–Gly (3–4) was also noted when the above incubated solution was examined by high performance liquid chromatography.

**Keywords**——porcine secretin solution synthesis; trifluoromethanesulfonic acid deprotection; $N^G$-mesitylenesulfonylarginine; Asp side reaction; secretin autolysis; secretin instability

The instability of secretin, a porcine gastrointestinal hormone, in aqueous solution is a well documented phenomenon.[2-7)] Succinimide formation followed by $\alpha \rightarrow \beta$ transpetidation at the Asp residue (position 3) in secretin has been postulated to explain this unusual phenomenon.[2,6-8)] Indeed, synthetic studies indicated that replacement of Asp (position 3) with other amino acids resulted in considerable loss of biological activity.[7,9)] However, the above proposal does not seem to be an adequate explanation, since a lack of parallelism between the rates of inactivation and succinimide formation was pointed out by Beyerman *et al.*[7)] Thus, besides transpeptidation, other subtle structural changes in the secretin molecule seem to be responsible for this phenomenon. Recently, we observed that the N-terminal His residue was partially hydrolyzed during incubation of the Met(O)-derivative of synthetic vasoactive intestinal polypeptide (VIP) with mercaptoethanol in an aqueous solution at pH 6.0[10)] and we predicted the possible hydrolysis of His from porcine secretin, which shares the identical N-terminal tripeptide unit, His–Ser–Asp, under similar conditions.

We have now synthesized porcine secretin in a different manner from those employed by other authors,[2,11-17)] and observed the liberation of $His^1$ from the synthetic peptide when it was incubated at 37 °C. At the same time, we observed that the hydrolysis of Asp-bonds of the synthetic peptide (positions 3 and 15) did indeed occur during the incubation. A detailed account of these investigations is presented herein.

Porcine secretin was synthesized in a conventional manner by assembling four peptide fragments according to the scheme illustrated in Fig. 1. In order to avoid the risk of base-catalyzed succinimide formation of the Asp residues (positions 3 and 15),[18-20)] the side chain protecting group was removed at the stage of each fragment synthesis. In contrast to previous

syntheses of secretin, we employed Arg(Mts),[21] the protecting group of which is known to be cleaved smoothly by treatment with 1 M TFMSA–thioanisole in TFA.[22,23] Certain advantageous features of this deprotecting procedure, including a decreased tendency for acid-catalyzed succinimide formation of the Asp residue, were recently reviewed.[24]

First, the protected C-terminal tetradecapeptide [1], Z(OMe)–Arg(Mts)–Asp–Ser(Bzl)–Ala–Arg(Mts)–Leu–Gln–Arg(Mts)–Leu–Leu–Gln–Gly–Leu–Val–$NH_2$, was prepared according to the scheme shown in Fig. 2. The active ester procedure[25] was employed for introduction of the corresponding amino acids and the azide procedure[26] for the four dipeptide hydrazides. In particular, after introduction of the Boc–Asp(OBu$^t$)–OH into the chain, the Bu$^t$ group was removed by TFA treatment, together with the Boc group, prior to the subsequent condensation for the reason stated above.

Fragment [2], Z(OMe)–Ser–Arg(Mts)–Leu–$NHNH_2$, was prepared by the azide condensation of Z(OMe)–Ser–$NHNH_2$ with a TFA-treated sample of the Z(OMe)–Arg(Mts)–Leu–OMe, which was synthesized in the preparation of fragment [1], followed by the usual hydrazine treatment.

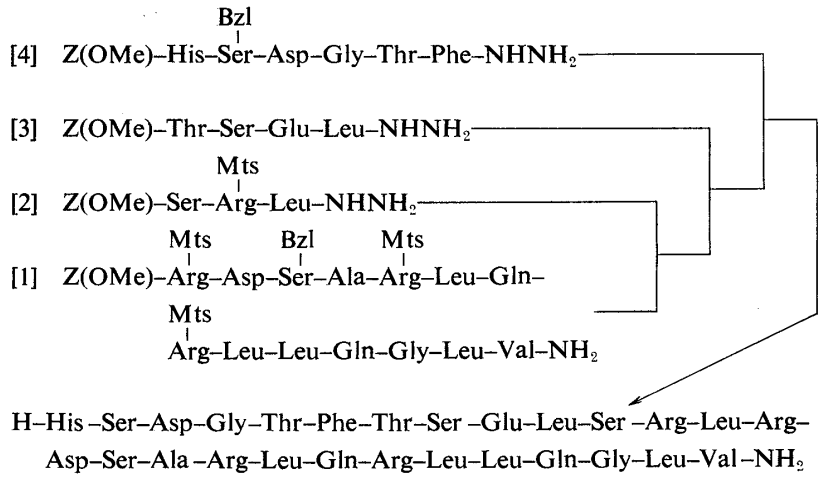


Fig. 1. Synthetic Route to Porcine Secretin

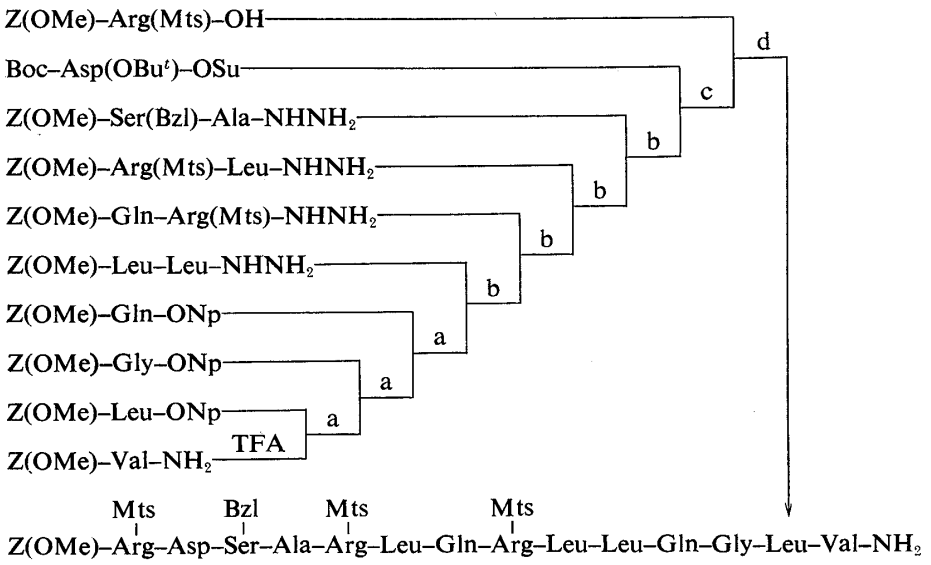


Fig. 2. Synthetic Scheme for the Protected Tetradecapeptide Amide, Z(OMe)–(pS 14—27)–$NH_2$ [1]

a: 1. Np, 2. TFA; b: 1. Azide, 2. TFA; c: 1. Su, 2. TFA; d: DCC-DNp.

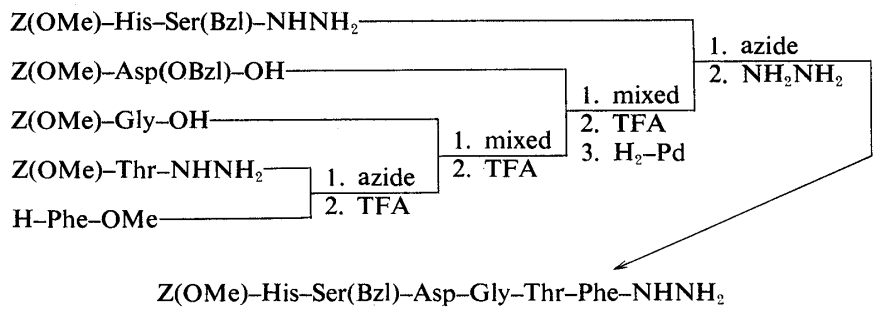


Fig. 3. Synthetic Scheme for the Protected Hexapeptide Hydrazide, Z(OMe)–(pS 1—6)–$NHNH_2$ [4]

TABLE I. Amino Acid Ratios in 6 N HCl Hydrolysates of Synthetic Porcine Secretin and Intermediates

| Position | Protected intermediates | | | | Synthetic porcine secretin |
|---|---|---|---|---|---|
| | 14—27 (14) | 11—27 (17) | 7—27 (21) | 1—27 (27) | |
| His | | | | 0.94 (1) | 0.95 (1) |
| Ser | 0.89 (1) | 1.87 (2) | 2.81 (3) | 3.40 (4) | 3.66 (4) |
| Asp | 0.94 (1) | 0.95 (1) | 0.93 (1) | 1.86 (2) | 2.03 (2) |
| Gly | 1.04 (1) | 1.02 (1) | 1.02 (1) | 2.01 (2) | 2.08 (2) |
| Thr | | | 1.14 (1) | 2.00 (2) | 1.90 (2) |
| Phe | | | | 1.21 (1) | 0.96 (1) |
| Glu | 2.06 (2) | 2.06 (2) | 3.16 (3) | 2.99 (3) | 3.09 (3) |
| Leu | 3.84 (4) | 4.93 (5) | 5.89 (6) | 6.10 (6) | 6.03 (6) |
| Arg | 2.81 (3) | 3.80 (4) | 3.78 (4) | 3.82 (4) | 4.25 (4) |
| Ala | 1.08 (1) | 1.07 (1) | 1.01 (1) | 1.01 (1) | 1.02 (1) |
| Val | 1.00 (1) | 1.00 (1) | 1.00 (1) | 1.00 (1) | 1.00 (1) |
| Recovery (%) | 92 | 86 | 88 | 90 | 83 |

Fragment [3], Z(OMe)–Thr–Ser–Glu–Leu–$NHNH_2$, was prepared as follows. First, Z(OMe)–Glu(OBzl)–Leu–OMe was prepared by the Np method[25] and the Bzl and Z(OMe) groups were removed by hydrogenolysis followed by TFA treatment, in order to make the preparation of the hydrazide easier. The resulting dipeptide ester, H–Glu–Leu–OMe, was coupled with Z(OMe)–Thr–Ser–$NHNH_2$[27] by the azide method to give Z(OMe)–Thr–Ser–Glu–Leu–OMe, which was converted to the corresponding hydrazide in the usual manner.

The N-terminal fragment [4], Z(OMe)–His–Ser(Bzl)–Asp–Gly–Thr–Phe–$NHNH_2$, was prepared as shown in Fig. 3. Z(OMe)–Asp(OBzl)–Gly–Thr–Phe–OMe was prepared in a stepwise manner starting with H–Phe–OMe, then the Z(OMe) group was first removed by TFA treatment and the Bzl ester group by hydrogenolysis. Referring to our previous synthesis of VIP,[28] Z(OMe)–His–Ser(Bzl)–$NHNH_2$ was employed instead of Z(OMe)–His–Ser–$NHNH_2$, since the latter gave a poor coupling with the amino component containing the Asp residue with the unprotected carboxyl group for some reason. The protected hexapeptide ester prepared in this way was converted to the corresponding hydrazide in the usual manner.

The four fragments thus prepared were condensed successively by the azide procedure to construct the heptacosapeptide amide chain of porcine secretin. Each fragment condensation was performed in DMF or a mixture of DMF and DMSO using 4 to 7 eq of the appropriate acyl component and each product was purified by gel-filtration on Sephadex LH-20. The

purity of every intermediate was assessed by thin-layer chromatography (TLC), elemental analysis and amino acid analysis. In the latter case, the C-terminal Val was selected as a diagnostic amino acid. Thus, by comparison of the recovery of Val with those of newly incorporated amino acids after each condensation, satisfactory condensation of each fragment could be ascertained.

Next, the protected heptacosapeptide amide was treated with 1 M TFMSA–thioanisole in TFA in the presence of *m*-cresol[29] in an ice-bath for 60 min to remove all protecting groups. This treatment was repeated twice more to ensure complete deprotection. The deprotected peptide was converted to the corresponding acetate with Amberlite IR-400 and treated with dil. ammonia in order to reverse a possible N→O shift[30] at the Ser and Thr residues. The treated product was purified by gel-filtration on Sephadex G-25 with 3% AcOH as an eluant, followed by ion-exchange chromatography on SP-Sephadex C-25 using gradient elution with pH 8.5, 0.1 M $KH_2PO_4$–NaOH buffer. For further purification, reverse phase-high performance liquid chromatography (RP-HPLC) on a Chemopak column was found to be extremely effective to remove some impurities. After gel-filtration, a highly purified product was obtained, the purity of which was confirmed by amino acid analyses after acid hydrolysis and enzymatic digestion. Especially satisfactory recovery of Asp in the enzymatic digestion was obtained, indicating that we had obtained synthetic secretin with a high degree of homogeneity free from contamination by the succinimide derivative. In terms of stimulation of the pancreatic alkaline juice flow in rats, our synthetic secretin was as active as an authentic sample of secretin purchased from the Peptide Institute Inc.

Next, using this synthetic secretin, we examined its behavior in an aqueous solution at 37 °C under two different conditions, *i.e.*, in slightly acidic solution (pH 5.0) and in slightly basic solution (pH 7.73). We observed, after 30 d, the release of His in *ca.* 3% yield on a molar basis under both conditions. Bodanszky *et al.*[31] and Beyerman *et al.*[32] previously pointed out the critical importance of the N-terminal His residue of secretin for its biological activity, since the activity of des-His[1]-secretin was found to be less than 1% of that of native secretin, while the structurally related compound VIP behaved in a biologically different manner from secretin. From the results cited above, it appears likely that besides the Asp-rearrangement

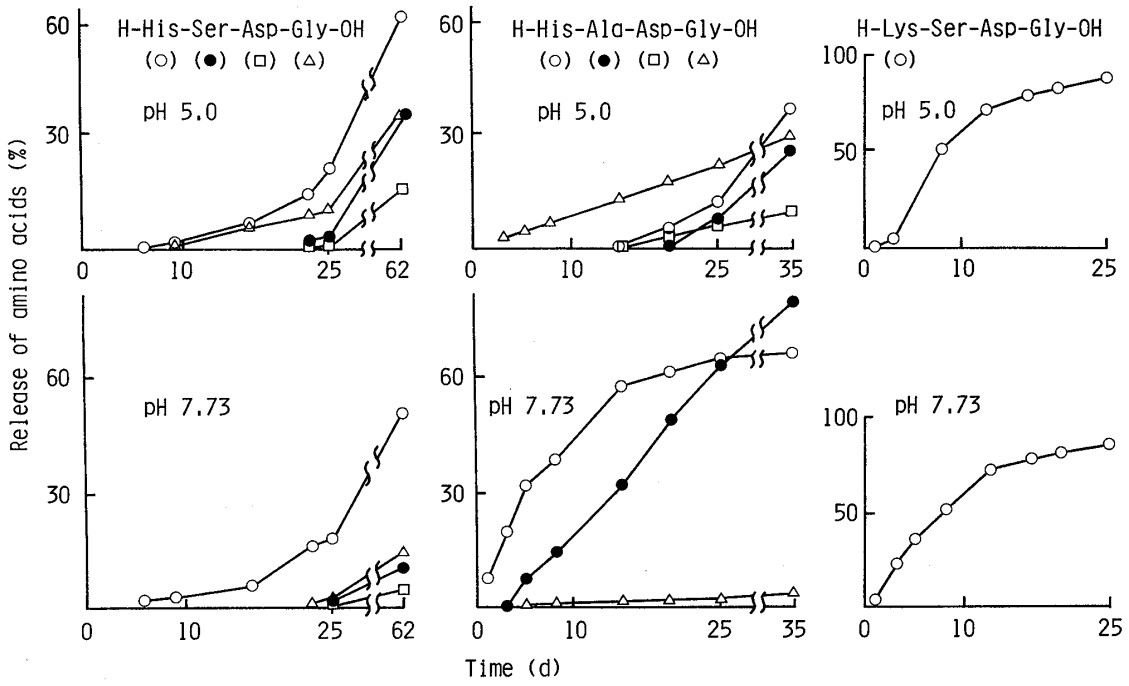


Fig. 4. Time Courses of the Hydrolysis of the Model Tetrapeptides at 37 °C

proposed by others, the unusual His-release is another reason for the instability of secretin in aqueous solution.

As described earlier, the N-terminal tripeptide sequence of secretin and VIP, His–Ser–Asp, is a combination of the triad amino acids of serine-protease.[33] We have preliminarily observed apparent autolysis behavior of the N-terminal tetrapeptide of VIP, His–Ser–Asp–Ala.[34] Thus, we decided to examine the role of the functional groups within the N-terminal tetrapeptide sequence of secretin, His–Ser–Asp–Gly (I), by using substituted analogs.

In addition to (I), five analogs, His–Ala–Asp–Gly (II), Lys–Ser–Asp–Gly (III), His–Ser–Asn–Gly (IV), His–Ser–Glu–Gly (V), and Ala–Ser–Asp–Gly (VI), were synthesized and these peptides were incubated at 37 °C in aqueous solutions of pH 5.0 and 7.73. The release of His from (I) and (II), and the release of Lys from (III) were observed within 15 d, as shown in Fig. 4.

Although little effect of pH was seen, except in the case of (II), these hydrolytic rates seem to be much faster than that of secretin, indicating that the $His^1$–$Ser^2$ bond of secretin itself is stabilized to some extent by the rest of the molecule. It is interesting to note that, together with the His release, successive fragmentation of peptide bonds was observed in (I), whereas no

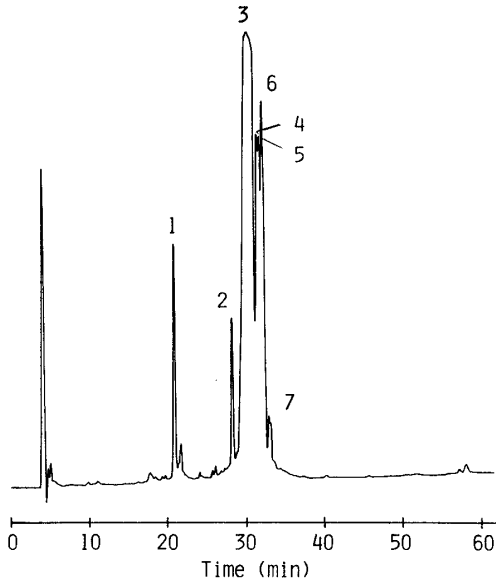


Fig. 5. HPLC Examination of Incubated Synthetic Porcine Secretin

TABLE II. Recoveries of Amino Acids in Acid Hydrolysates of Products Formed during Incubation of Synthetic Porcine Secretin

| | Peak No. | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| His (1) | 0.90 | | 0.89 | 0.70 | 0.79 | 0.31 | |
| Ser (4) | 2.71 | 1.07 | 4.06 | 3.34 | 3.38 | 2.73 | 1.38 |
| Asp (2) | 1.74 | | 1.94 | 1.69 | 1.69 | 1.11 | 0.69 |
| Gly (2) | 1.00 | 1.05 | 1.97 | 1.83 | 1.84 | 1.89 | 1.09 |
| Thr (2) | 1.88 | | 2.03 | 1.86 | 1.86 | 1.83 | 0.85 |
| Phe (1) | 1.45 | | 0.93 | 1.16 | 1.29 | 1.08 | |
| Glu (3) | 1.01 | 1.98 | 3.09 | 2.82 | 2.83 | 2.76 | 1.27 |
| Leu (6) | 1.92 | 4.02 | 6.33 | 5.68 | 5.53 | 5.22 | 3.35 |
| Arg (4) | 1.55 | 1.90 | 4.08 | 3.42 | 3.45 | 3.23 | 0.89 |
| Ala (1) | | 0.98 | 1.04 | 0.97 | 0.97 | 1.05 | 0.74 |
| Val (1) | | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |

release of His was observed from the analogs, (IV), (V) and (VI), which do not have either Asp or a basic amino acid within the sequence. These experimental data seem to justify the conclusion that the combination of the basic amino acid (His) at position 1 and the $\beta$-carboxyl group of Asp at position 3 is responsible for the apparent autolysis of the N-terminal portion of secretin, particularly the release of His.

In the above experiments, the release of Gly from (I) seems to proceed through a succinimide intermediate, the formation of which was suggested by Beyerman *et al.*[7] Since secretin possesses two Asp residues (positions 3 and 15), we examined the possible hydrolysis of synthetic secretin at these two positions. When a solution of synthetic secretin incubated at pH 5.0 (30 d) was examined by HPLC, besides the parent peak corresponding to synthetic secretin, more than 6 other peaks were detected (Fig. 5). Acid hydrolysis (Table II) indicated that, of these, peaks 1 and 2 correspond to the N-terminal pentadecapeptide and the C-terminal dodecapeptide produced by hydrolysis of the Asp–Ser bond (15–16) of secretin, respectively. Peaks 4, 5 and 6 were not well separated, but low recoveries of His, Ser and Asp were noted in these peaks, particularly in peak 6. It thus appeared that these peaks were due to the release of His, Ser and Asp in addition to the hydrolysis of the Asp peptide bond (3–4) of secretin. It is interesting to note that the Asp–Gly and the Asp–Ser bonds (known to be susceptible to succinimide formation) are the sites of autolysis of secretin in aqueous solution.

Thus, the major causes of inactivation of secretin in solution are considered to be release of His and hydrolysis of the Asp bond. These results imply that any peptide that contains such an Asp–X (X = Gly or Ser) bond may suffer autolysis when stored in aqueous solution.

## Experimental

Unless otherwise stated, the following experimental procedures were employed in this investigation.

$N^{\alpha}$-Deprotection: The $N^{\alpha}$-protecting group, Z(OMe) or Boc, was cleaved with TFA (*ca.* 2 ml per 1 g of a peptide) in the presence of anisole (2 mol eq or more) at ice-bath temperature for 60 min. After evaporation of the TFA *in vacuo* at 30 °C or less, the residue was treated with dry ether. If a powder was obtained, it was collected by filtration, dried over KOH pellets *in vacuo* for 3 h and used for the coupling reaction. If an oily precipitate was obtained, it was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 3 h and used for the coupling reaction.

Condensation Reactions: The DCC[35] and active ester[25] condensations were carried out at room temperature (17—25 °C). Each hydrazide was converted to the corresponding azide by treatment with isoamyl nitrite,[26] and the azide condensation was carried out at 4 °C. Mixed anhydride[36,37] was prepared by using isobutyl chloroformate.

Purification: Unless otherwise mentioned, products were purified by one of the following procedures. Procedure A: The protected peptide was dissolved in AcOEt, then the solution was washed with 5% citric acid, 5% $NaHCO_3$ and NaCl–$H_2O$, dried over $Na_2SO_4$ and concentrated. The residue was crystallized or precipitated from appropriate solvents. Procedure B: The protected peptide was triturated with ether and 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and $H_2O$, then crystallized or precipitated from appropriate solvents.

TLC was performed on silica gel (Kiesel gel, Merck). *Rf* values refer to the following solvent systems: $Rf_1$ $CHCl_3$–MeOH–$H_2O$ (8:3:1), $Rf_2$ $CHCl_3$–MeOH–AcOH (9:3:0.5), $Rf_3$ $CHCl_3$–MeOH–AcOH (9:1:0.5), $Rf_4$ $CHCl_3$–MeOH (20:1), $Rf_5$ *n*-BuOH–AcOH–Pyridine–$H_2O$ (4:1:1:2), $Rf_6$ $CHCl_3$–MeOH (40:1), $Rf_7$ *n*-BuOH–AcOEt–AcOH–$H_2O$ (1:1:1:1).

RP-HPLC was conducted with a Waters M45 compact model coupled to a Chemopak column (Nucleosil 7C18, 4.6 × 250 mm). As a standard sample, synthetic porcine secretin was purchased from the peptide Institute Inc., Minoh, Osaka 562 (Lot. No. 340530).

### 1. Synthesis of Porcine Secretin

**Z(OMe)–Leu–Val–$NH_2$**——A mixture of H–Val–$NH_2$ [prepared from 12.25 g of the Z(OMe)-derivative by the usual TFA treatment], $Et_3N$ (12.6 ml, 91.8 mmol) and Z(OMe)–Leu–ONp (20.02 g, 48.1 mmol) in DMF (150 ml) was stirred overnight, then the solution was concentrated and the residue was treated with 5% citric acid and ether. The resulting powder was purified by procedure B, followed by recrystallization from MeOH–ether; yield 14.80 g (86%), mp 214—216 °C, $[\alpha]_D^{20}$ +4.5° ($c$ = 0.4, DMF), $Rf_1$ 0.68. *Anal.* Calcd for $C_{20}H_{31}N_3O_5$: C, 61.05; H, 7.94; N, 10.68. Found: C, 61.29; H, 8.05; N, 10.72.

**Z(OMe)–Gly–Leu–Val–$NH_2$**——A TFA-treated sample of Z(OMe)–Leu–Val–$NH_2$ (14.95 g, 38.0 mmol) was dissolved in DMF (150 ml), together with $Et_3N$ (11.0 ml, 79.8 mmol) and Z(OMe)–Gly–ONp (15.06 g, 41.8 mmol). After being stirred overnight, the solution was concentrated and the resulting powder was purified by procedure B,

followed by recrystallization from MeOH–ether; yield 21.11 g (90%), mp 202—204 °C, $[\alpha]_D^{20}$ −14.8° ($c$=0.5, DMF), $Rf_1$ 0.59. *Anal.* Calcd for $C_{22}H_{34}N_4O_6$: C, 58.64; H, 7.61; N, 12.44. Found: C, 58.51; H, 7.64; N, 12.30.

**Z(OMe)–Gln–Gly–Leu–Val–NH$_2$**——A TFA-treated sample of Z(OMe)–Gly–Leu–Val–$NH_2$ (3.11 g, 6.90 mmol) was dissolved in DMF–DMSO (2 : 1, 40 ml), together with $Et_3N$ (2.0 ml, 14.49 mmol) and Z(OMe)–Gln–ONp (3.28 g, 7.59 mmol). After being stirred overnight, the solution was concentrated and the resulting product was purified by procedure B, followed by precipitation from DMF–AcOEt; yield 3.60 g (90%), mp 223—225 °C, $[\alpha]_D^{20}$ −15.4° ($c$=0.5, DMF), $Rf_1$ 0.55. *Anal.* Calcd for $C_{27}H_{42}N_6O_8$: C, 56.04; H, 7.32; N, 14.52. Found: C, 55.77; H, 7.38; N, 14.45.

**Z(OMe)–Leu–Leu–NHNH$_2$**——Z(OMe)–Leu–OH (14.77 g, 50 mmol) and H–Leu–OMe [prepared from 10.90 g (60 mmol) of the hydrochloride] in DMF (70 ml) were condensed by the use of DCC in the usual manner. The oily product purified by procedure A was dissolved in MeOH and treated with 80% hydrazine hydrate (30 ml, 10 eq) at room temperature overnight. The solvent was removed by evaporation and the residue was treated with $H_2O$ to afford a powder, which was recrystallized from MeOH–*n*-hexane; yield 10.46 g (50%), mp 149—153 °C, $[\alpha]_D^{20}$ −40.5° ($c$=0.6, MeOH), $Rf_1$ 0.68. *Anal.* Calcd for $C_{21}H_{34}N_4O_5$: C, 59.69; H, 8.12; N, 13.26. Found: C, 59.71; H, 8.21; N, 13.20.

**Z(OMe)–Leu–Leu–Gln–Gly–Leu–Val–NH$_2$**——A TFA-treated sample of Z(OMe)–Gln–Gly–Leu–Val–$NH_2$ (3.55 g, 6.13 mmol) was dissolved in DMF (40 ml) containing NMM (0,67 ml, 6.13 mmol). The azide [prepared from 3.89 g (9.20 mmol) of Z(OMe)–Leu–Leu–$NHNH_2$] in DMF (40 ml) and NMM (1.01 ml, 9.20 mmol) were added to the above ice-chilled solution. The mixture was stirred at 4 °C for 24 h and then 5% citric acid (150 ml) was added to give a powder, which was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 4.64 g (95%), mp 242—245 °C, $[\alpha]_D^{20}$ −19.8° ($c$=0.5, DMF), $Rf_1$ 0.60. Amino acid ratios in 6 N HCl hydrolysate: Glu 1.07, Gly 0.98, Leu 3.09, Val 1.00 (recovery 98%). *Anal.* Calcd for $C_{39}H_{64}N_8O_{10}$: C, 58.19; H, 8.01; N, 13.92. Found: C, 57.96; H, 8.23; N, 13.65.

**Z(OMe)–Gln–Arg(Mts)–NHNH$_2$**——Z(OMe)–Arg(Mts)–OH [prepared from 30.99 g (50 mmol) of the CHA salt] was methylated with $CH_2N_2$, then treated with TFA as usual. The resulting methyl ester was dissolved in DMF (250 ml), together with Z(OMe)–Gln–ONp (25.88 g, 60 mmol) and $Et_3N$ (16.6 ml, 120 mmol). After being stirred overnight, the solution was concentrated. The oily dipeptide methyl ester isolated by procedure A was dissolved in MeOH (300 ml) and treated with 80% hydrazine hydrate (30 ml, 10 eq ) for 48 h. The solvent was removed by evaporation and the residue was treated with $H_2O$ to form a powder, which was recrystallized from MeOH–ether; yield 23.32 g (70%), mp 152—155 °C, $[\alpha]_D^{25}$ −4.3° ($c$=0.5, DMF), $Rf_1$ 0.62, $Rf_3$ 0.28. *Anal.* Calcd for $C_{29}H_{42}N_8O_8S$: C, 52.55; H, 6.39; N, 16.91. Found: C, 52.26; H, 6.51; N, 16.75.

**Z(OMe)–Gln–Arg(Mts)–Leu–Leu–Gln–Gly–Leu–Val–NH$_2$**——A TFA-treated sample of Z(OMe)–Leu–Leu–Gln–Gly–Leu–Val–$NH_2$ (10.14 g, 12.60 mmol) was dissolved in DMF (80 ml) containing $Et_3N$ (1.74 ml, 12.60 mmol). The azide [prepared from 10.02 g (15.12 mmol) of Z(OMe)–Gln–Arg(Mts)–$NHNH_2$] in DMF (70 ml) and $Et_3N$ (2.09 ml, 15.12 mmol) were added to the above ice-chilled solution. After being stirred at 4 °C overnight, the whole was poured into 5% citric acid (150 ml) to give a powder, which was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 13.55 g (85%), mp 257—259 °C, $[\alpha]_D^{25}$ −22.3° ($c$=0.6, DMF), $Rf_1$ 0.42. Amino acid ratios in 6 N HCl hydrolysate: Glu 2.10, Gly 0.99, Leu 3.00, Arg 0.96, Val 1.00 (recovery 97%). *Anal.* Calcd for $C_{59}H_{94}N_{14}O_{15}S$: C, 55.73; H, 7.45; N, 15.42. Found: C, 55.49; H, 7.70; N, 15.46.

**Z(OMe)–Arg(Mts)–Leu–OMe**——A mixed anhydride of Z(OMe)–Arg(Mts)–OH [prepared from 16.20 g (26.1 mmol) of the CHA salt] was condensed with H–Leu–OMe [prepared from 5.22 g (28.8 mmol) of the hydrochloride] in DMF (60 ml). The product was isolated by procedure A and recrystallized from isopropyl ether and ether (1 : 1); yield 13.90 g (82%), mp 55—56 °C, $[\alpha]_D^{25}$ −23.8° ($c$=1.0, MeOH), $Rf_1$ 0.83. *Anal.* Calcd for $C_{31}H_{45}N_5O_8S$: C, 57.48; H, 7.00; N, 10.81. Found: C, 57.53; H, 7.13; N, 10.83.

**Z(OMe)–Arg(Mts)–Leu–NHNH$_2$**——Z(OMe)–Arg(Mts)–Leu–OMe (10.0 g, 15.4 mmol) in MeOH (100 ml) was treated with 80% hydrazine hydrate (2.81 ml, 46.3 mmol) at room temperature for 24 h. The solvent was removed by evaporation and the residue was treated with $H_2O$ to form a powder, which was recrystallized from MeOH with ether; yield 7.61 g (76%), mp 119—121 °C, $[\alpha]_D^{25}$ −15.5° ($c$=0.6, MeOH), $Rf_1$ 0.65. *Anal.* Calcd for $C_{30}H_{45}N_7O_7S$: C, 55.62; H, 7.00; N, 15.14. Found: C, 55.90; H, 7.08; N, 15.19.

**Z(OMe)–Arg(Mts)–Leu–Gln–Arg(Mts)–Leu–Leu–Gln–Gly–Leu–Val–NH$_2$**——A TFA-treated sample of Z(OMe)–Gln–Arg(Mts)–Leu–Leu–Gln–Gly–Leu–Val–$NH_2$ (4.47 g, 3.52 mmol) was dissolved in DMF–DMSO (1 : 1, 50 ml) containing NMM (0.39 ml, 3.52 mmol). The azide [prepared from 2.97 g (4.58 mmol) of Z(OMe)–Arg(Mts)–Leu–$NHNH_2$] in DMF (40 ml) and NMM (0.50 ml, 4.59 mmol) were added to the above ice-chilled solution and the reaction mixture, after being stirred at 4 °C for 48 h, was concentrated. Treatment of the residue with 5% citric acid afforded a powder, which was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 5.11 g (85%), mp 242—244 °C, $[\alpha]_D^{25}$ −8.9° ($c$=0.6, DMF), $Rf_1$ 0.53. Amino acid ratios in 6 N HCl hydrolysate: Glu 2.08, Gly 1.02, Leu 3.82, Arg 1.84, Val 1.00 (recovery 94%). *Anal.* Calcd for $C_{80}H_{127}N_{19}O_{19}S \cdot H_2O$: C, 55.18; H, 7.47; N, 15.29. Found: C, 55.14; H, 7.29; N, 15.13.

**Z(OMe)–Ser(Bzl)–OMe**——Z(OMe)–Ser(Bzl)–OH (12.60 g, 35.0 mmol) was methylated with $CH_2N_2$ as usual and the product was recrystallized from MeOH and isopropyl ether; yield 13.0 g (99%), mp 39—40 °C, $[\alpha]_D^{25}$ −10.5°

($c=1.1$, MeOH), $Rf_1$ 0.88. *Anal.* Calcd for $C_{20}H_{23}NO_6$: C, 64.33; H, 6.21; N, 3.75. Found: C, 64.26; H, 6.25; N, 4.00.

**Z(OMe)–Ser(Bzl)–NHNH$_2$**——Z(OMe)–Ser(Bzl)–OMe (12.80 g, 34.3 mmol) in MeOH (130 ml) was treated with 80% hydrazine hydrate (6.25 ml, 3 eq) overnight. The solid that formed on standing was precipitated from DMF with MeOH; yield 9.85 g (77%), mp 33—34 °C, $[\alpha]_D^{25}$ +8.0° ($c=1.1$, MeOH), $Rf_1$ 0.55. *Anal.* Calcd for $C_{22}H_{28}N_4O_6$: C, 61.11; H, 6.21; N, 11.25. Found: C, 61.32; H, 6.17; N, 11.24.

**Z(OMe)–Ser(Bzl)–Ala–OMe**——The azide [prepared from 8.55 g (22.9 mmol) of Z(OMe)–Ser(Bzl)–NHNH$_2$] in DMF (120 ml) was added to an ice-chilled solution of H–Ala–OMe [prepared from 3.84 g (27.5 mmol) of the hydrochloride] in DMF (40 ml). After addition of $Et_3N$ (3.16 ml, 22.9 mmol), the solution was stirred at 4 °C overnight and the solvent was removed by evaporation. The product was isolated by procedure A and recrystallized from MeOH and ether; yield 9.90 g (97%), mp 136—138 °C, $[\alpha]_D^{25}$ −5.3° ($c=0.9$, MeOH), $Rf_1$ 0.81. *Anal.* Calcd for $C_{23}H_{28}N_2O_7$: C, 62.15; H, 6.35; N, 6.30. Found: C, 62.35; H, 6.38; N, 6.39.

**Z(OMe)–Ser(Bzl)–Ala–NHNH$_2$**——Z(OMe)–Ser(Bzl)–Ala–OMe (9.0 g, 19.7 mmol) in MeOH (90 ml) was treated with 80% hydrazine hydrate (3.59 ml, 3 eq) for 4 h. The solid standing was collected and reprecipitated from DMF with MeOH; yield 7.69 g (88%), mp 164—165 °C, $[\alpha]_D^{25}$ +9.3° ($c=1.0$, DMF), $Rf_3$ 0.52. *Anal.* Calcd for $C_{22}H_{28}N_4O_6$: C, 59.45; H, 6.35; N, 12.61. Found: C, 59.37; H, 6.28; N, 12.73.

**Z(OMe)–Ser(Bzl)–Ala–Arg(Mts)–Leu–Gln–Arg(Mts)–Leu–Leu–Gln–Gly–Leu–Val–NH$_2$**——A TFA-treated sample of Z(OMe)–Arg(Mts)–Leu–Gln–Arg(Mts)–Leu–Leu–Gln–Gly–Leu–Val–NH$_2$ (3.0 g, 1.74 mmol) was dissolved in DMF (30 ml) containing $Et_3N$ (0.24 ml, 1.74 mmol). The azide [prepared from 0.93 g (2.09 mmol) of Z(OMe)–Ser(Bzl)–Ala–NHNH$_2$] in DMF (30 ml) and $Et_3N$ (0.29 ml, 2.09 mmol) were added to the above ice-chilled solution and the whole, after being stirred at 4 °C overnight, was concentrated. The product was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 2.93 g (85%), mp 255—257 °C, $[\alpha]_D^{28}$ −2.0° ($c=1.0$, DMF), $Rf_1$ 0.58. Amino acid ratios in 6 N HCl hydrolysate: Ser 0.85, Glu 2.04. Gly 1.00, Ala 0.86, Leu 3.82, Arg 1.83, Val 1.00 (recovery 80%). *Anal.* Calcd for $C_{93}H_{143}N_{21}O_{22}S \cdot 2H_2O$: C, 55.64; H, 7.38; N, 14.65. Found: C, 55.54; H, 7.28; N, 14.87.

**Boc–Asp(OBu$^t$)–Ser(Bzl)–Ala–Arg(Mts)–Leu–Gln–Arg(Mts)–Leu–Leu–Gln–Gly–Leu–Val–NH$_2$**——A TFA-treated sample of Z(OMe)–Ser(Bzl)–Ala–Arg(Mts)–Leu–Gln–Arg(Mts)–Leu–Leu–Gln–Gly–Leu–Val–NH$_2$ (6.41 g, 3.25 mmol) was dissolved in DMF–DMSO (1 : 1, 50 ml), together with $Et_3N$ (0.45 ml, 3.25 mmol), Boc–Asp(OBu$^t$)–OSu (2.51 g, 6.50 mmol) and NMM (0.72 ml, 6.50 mmol). The mixture was stirred at 4 °C overnight, then 5% citric acid (150 ml) was added to give a powder, which was washed with AcOEt and precipitated from DMF with AcOEt; yield 6.00 g (89%), mp 251—253 °C, $[\alpha]_D^{28}$ −3.1° ($c=1.0$, DMF), $Rf_2$ 0.85. Amino acid ratios in 6 N HCl hydrolysate: Asp 0.84, Ser 0.83, Gly 0.99, Glu 2.07, Leu 3.70, Arg 1.81, Ala 0.84. Val 1.00 (recovery 95%). *Anal.* Calcd for $C_{97}H_{156}N_{22}O_{24}S \cdot 2H_2O$: C, 55.09; H, 7.62; N, 14.57. Found: C, 55.10; H, 7.63; N, 14.76.

**Z(OMe)–Arg(Mts)–Asp–Ser(Bzl)–Ala–Arg(Mts)–Leu–Gln–Arg(Mts)–Leu–Leu–Gln–Gly–Leu–Val–NH$_2$ [Z(OMe)–(pS 14—27)–NH$_2$]**——The above protected tridecapeptide amide (6.88 g, 3.31 mmol) was treated with TFA at room temperature for 4 h, then the treated peptide was dissolved in DMF–DMSO (2 : 1, 50 ml), together with $Et_3N$ (0.46 ml, 3.31 mmol), Z(OMe)–Arg(Mts)–ODNp [prepared from 2.24 g (4.30 mmol) of Z(OMe)–Arg(Mts)–OH by the usual DCC procedure] in DMF (25 ml) and NMM (0.47 ml, 4.30 mmol) and the reaction mixture, after being stirred at 4 °C overnight, was poured into 5% citric acid (150 ml). The resulting powder was washed with AcOEt and precipitated from DMF with AcOEt; yield 6.55 g (82%), mp 250—252 °C, $[\alpha]_D^{28}$ −15.5° ($c=1.0$, DMF), $Rf_1$ 0.60. *Anal.* Calcd for $C_{112}H_{170}N_{26}O_{28}S_3$: C, 55.47; H, 7.07; N, 15.02. Found: C, 55.39; H, 7.23; N, 14.77.

**Z(OMe)–Ser–Arg(Mts)–Leu–NHNH$_2$ [Z(OMe)–(pS 11—13)–NHNH$_2$]**——A TFA-treated sample of Z(OMe)–Arg(Mts)–Leu–OMe (11.36 g, 17.54 mmol) was dissolved in DMF (80 ml) containing $Et_3N$ (2.42 ml, 17.54 mmol). The azide [prepared from 6.46 g (22.80 mmol) of Z(OMe)–Ser–NHNH$_2$] in DMF (70 ml) and $Et_3N$ (3.15 ml, 22.8 mmol) were added to the above ice-chilled solution and the reaction mixture, after being stirred at 4 °C for 24 h, was concentrated. The product was isolated by procedure B and purified by column chromatography on silica gel (4 × 30 cm) using $CHCl_3$–MeOH (5 : 1) as an eluant. The solvent of the desired fractions ($Rf_1$ 0.72) was evaporated off and the residue, after being dissolved in MeOH (60 ml), was treated overnight with 80% hydrazine hydrate (2.95 ml, 6 eq). The solvent was evaporated off and the residue was precipitated from *n*-BuOH with ether; yield 4.76 g (80%), mp 104—108 °C, $[\alpha]_D^{27}$ −6.4° ($c=0.9$, MeOH), $Rf_1$ 0.72. Amino acid ratios in 6 N HCl hydrolysate: Ser 0.94, Arg 1.02, Leu 1.00, (recovery 81%). *Anal.* Calcd for $C_{33}H_{50}N_8O_9S \cdot H_2O$: C, 52.64; H, 6.96; N, 14.88. Found: C, 52.40; H, 6.99; N, 15.18.

**Z(OMe)–Glu(OBzl)–Leu–OMe**——A mixture of Z(OMe)–Glu–(OBzl)–ONp (20.04 g, 38.36 mmol), H–Leu–OMe [prepared from 6.30 g (34.87 mmol) of the hydrochloride] and NMM (4.22 ml, 38.36 mmol) in DMF (100 ml) was stirred at 4 °C overnight, then the solvent was removed by evaporation. The product was isolated by procedure A and recrystallized from AcOEt with ether; yield 14.0 g (76%), mp 89—91 °C, $[\alpha]_D^{28}$ −26.8° ($c=0.9$, MeOH), $Rf_1$ 0.92, $Rf_4$ 0.39. *Anal.* Calcd for $C_{28}H_{36}N_2O_8$: C, 63.62; H, 6.87; N, 5.30. Found: C, 63.33; H, 6.88; N, 5.22.

**Z(OMe)–Thr–Ser–Glu–Leu–OMe**——Z(OMe)–Glu(OBzl)–Leu–OMe (6.61 g, 12.51 mmol) in MeOH–$H_2O$ (10 : 1, 70 ml) was hydrogenated over a Pd catalyst for 6 h. The mixture was filtered, the filtrate was concentrated, and the residue was treated with TFA–anisole (12 ml–4.1 ml). The resulting TFA salt ($Rf_1$ 0.63), isolated as usual, was dissolved in DMF (50 ml) containing NMM (1.38 ml, 12.51 mmol). The azide [prepared from 7.21 g (18.77 mmol) of

Z(OMe)–Thr–Ser–$NHNH_2$] in DMF (80 ml) and NMM (2.06 ml, 18.77 mmol) were added to the above ice-chilled solution and the reaction mixture, after being stirred at 4 °C overnight, was concentrated. The product was isolated by procedure B and recrystallized from MeOH with AcOEt; yield 7.18 g (92%), mp 134—136 °C, $[\alpha]_D^{28}$ −3.0° (*c* = 1.0, DMF), $Rf_1$ 0.31. *Anal.* Calcd for $C_{28}H_{42}N_4O_{12} \cdot 1/2H_2O$: C, 52.90; H, 6.82; N, 8.81. Found: C, 52.93; H, 6.69; N, 8.93.

**Z(OMe)–Thr–Ser–Glu–Leu–$NHNH_2$ [Z(OMe)–(pS 7—10)–$NHNH_2$]**——Z(OMe)–Thr–Ser–Glu–Leu–OMe (6.25 g, 10.01 mmol) in MeOH (70 ml) was treated with 80% hydrazine hydrate (1.82 ml, 3 eq) for 48 h and the solvent was removed by evaporation. The resulting solid was precipitated twice from DMF with MeOH; yield 5.01 g (80%), mp 197—199 °C, $[\alpha]_D^{28}$ −1.1° (*c* = 0.9, DMSO), $Rf_5$ 0.78. Amino acid ratios in 6 N HCl hydrolysate: Thr 0.99, Ser 0.95, Glu 1.04, Leu 1.00 (recovery 88%). *Anal.* Calcd for $C_{27}H_{42}N_6O_{11}$: C, 51.75; H, 6.76; N, 13.41. Found: C, 51.51; H, 6.89; N, 13.56.

**Z(OMe)–Gly–Thr–Phe–OMe**——A TFA-treated sample of Z(OMe)–Thr–Phe–OMe (10.65 g, 23.96 mmol) was dissolved in DMF (70 ml) containing $Et_3N$ (3.31 ml, 23.96 mmol). A mixed anhydride [prepared from 6.88 g (28.75 mmol) of Z(OMe)–Gly–OH] in DMF (70 ml) was added to the above ice-chilled solution and the reaction mixture, after being stirred in an ice-bath for 2 h, was concentrated. The product was isolated by procedure B and recrystallized from MeOH with ether; yield 9.65 g (80%), mp 35—36 °C, $[\alpha]_D^{25}$ +10.1° (*c* = 1.0, MeOH), $Rf_1$ 0.80, $Rf_3$ 0.59. *Anal.* Calcd for $C_{25}H_{31}N_3O_8 \cdot 1/2H_2O$: C, 58.81; H, 6.32; N, 8.23. Found: C, 58.77; H, 6.00; N, 8.52.

**Z(OMe)–Asp(OBzl)–Gly–Thr–Phe–OMe**——A TFA-treated sample of Z(OMe)–Gly–Thr–Phe-OMe (8.53 g, 17.01 mmol) was dissolved in DMF (50 ml) containing $Et_3N$ (2.35 ml, 17.01 mmol). A mixed anhydride [prepared from 7.91 g (20.41 mmol) of Z(OMe)–Asp(OBzl)–OH] in DMF (80 ml) was added to the above ice-chilled solution and the reaction mixture, after being stirred in an ice-bath for 3 h, was concentrated. The product was isolated by procedure B and precipitated from DMF with MeOH; yield 9.48 g (79%), mp 189—191 °C, $[\alpha]_D^{25}$ −12.1° (*c* = 1.1, DMF), $Rf_3$ 0.70. *Anal.* Calcd for $C_{36}H_{42}N_4O_{11}$: C, 61.18; H, 5.99; N, 7.93. Found: C, 61.08; H, 5.90; N, 7.91.

**Z(OMe)–His–Ser(Bzl)–Asp–Gly–Thr–Phe–OMe**——A TFA-treated sample of Z(OMe)–Asp(OBzl)–Gly–Thr–Phe–OMe (5.00 g, 7.07 mmol) was dissolved in DMF (50 ml) and hydrogenated over a Pd catalyst for 4 h. The mixture was filtered, and the filtrate was neutralized with NMM (0.78 ml, 7.07 mmol). The azide [prepared from 3.61 g (8.48 mmol) of Z(OMe)–His–Ser(Bzl)–$NHNH_2$] in DMF (40 ml) and NMM (0.93 ml, 8.48 mmol) were added to the above ice-chilled filtrate and the reaction mixture, after being stirred at 4 °C overnight, was concentrated. The product was isolated by procedure B and precipitated from DMF with AcOEt; yield 4.21 g (64%), mp 164—165 °C, $[\alpha]_D^{25}$ −15.6° (*c* = 1.0, DMF), $Rf_1$ 0.28. *Anal.* Calcd for $C_{45}H_{54}N_8O_{14}$: C, 58.05; H, 5.85; N, 12.04. Found: C, 57.89; H, 6.00; N, 11.95.

**Z(OMe)–His–Ser(Bzl)–Asp–Gly–Thr–Phe–$NHNH_2$ [Z(OMe)–(pS 1—6)–$NHNH_2$]**——The above protected hexapeptide ester (3.51 g, 3.76 mmol) in DMF (40 ml) was treated with 80% hydrazine hydrate (0.69 ml, 3 eq), and the solid, that formed on standing overnight was precipitated from DMF with MeOH; yield 2.77 g (79%), mp 175—176 °C, $[\alpha]_D^{27}$ −9.9° (*c* = 1.0, DMF), $Rf_1$ 0.20. Amino acid ratios in 6 N HCl hydrolysate: His 0.94, Ser 0.94, Asp 0.98, Gly 1.05, Thr 0.99, Phe 1.00 (recovery 83%). Amino acid ratios in leucine aminopeptidase (LAP) digest of the TFA-treated sample: His 0.95, Ser(Bzl) + Phe 1.96 (calcd. as Phe), Asp 0.95, Gly 1.01, Thr 1.00 (recovery of Phe 77%). *Anal.* Calcd for $C_{44}H_{54}N_{10}O_{13}$: C, 56.76; H, 5.85; N, 15.05. Found: C, 56.61; H, 6.11; N, 14.72.

**Z(OMe)–Ser–Arg(Mts)–Leu–Arg(Mts)–Asp–Ser(Bzl)–Ala–Arg(Mts)–Leu–Gln–Arg(Mts)–Leu–Leu–Gln–Gly–Leu–Val–$NH_2$ [Z(OMe)–(pS 11—27)–$NH_2$]**——A TFA-treated sample of Z(OMe)–(pS 14—27)–$NH_2$ (4.75 g, 1.96 mmol) was dissolved in DMF–DMSO (2 : 1, 70 ml) containing $Et_3N$ (0.27 ml, 1.96 mmol). The azide [prepared from 7.29 g (9.80 mmol) of Z(OMe)–(pS 11—13)–$NHNH_2$] in DMF (70 ml) and NMM (1.08 ml, 9.80 mmol) were added to the above ice-chilled solution and the reaction mixture was stirred at 4 °C for 24 h. Additional azide [prepared from 1.44 g (1 eq) of the hydrazide] in DMF (15 ml) and NMM (0.22 ml, 1 eq) were added and stirring was continued for 24 h. The solvent was removed by evaporation and 5% citric acid (50 ml) was added. The resulting powder was washed with $H_2O$ and purified by gel-filtration on Sephadex LH-20 (2.5 × 130 cm) using DMF as an eluant. Individual fractions (9 ml each) were examined by measurement of the ultraviolet (UV) absorption at 270 nm, and the solvent of the desired fractions (tube Nos. 32—37) was removed by evaporation. The residue was precipitated from DMF–DMSO (4 : 1) with AcOEt; yield 3.52 g (61%), mp 220—223 °C, $[\alpha]_D^{25}$ −17.9° (*c* = 1.0, DMSO), $Rf_1$ 0.59. *Anal.* Calcd for $C_{136}H_{208}N_{32}O_{34}S_4 \cdot 4H_2O$: C, 53.81; H, 7.17; N, 14.76. Found: C, 54.06; H, 7.10; N, 14.45.

**Z(OMe)–Thr–Ser–Glu–Leu–Ser–Arg(Mts)–Leu–Arg(Mts)–Asp–Ser(Bzl)–Ala–Arg(Mts)–Leu–Gln–Arg(Mts)–Leu–Leu–Gln–Gly–Leu–Val–$NH_2$ [Z(OMe)–(pS 7—27)–$NH_2$]**——A TFA-treated sample of Z(OMe)–(pS 11—27)–$NH_2$ (2.62 g, 0.88 mmol) was dissolved in DMF–DMSO (2 : 1, 20 ml) containing $Et_3N$ (122 μl, 0.88 mmol). The azide [prepared from 1.66 g (2.65 mmol) of Z(OMe)–(pS 7—10)–$NHNH_2$] in DMF (17 ml) and NMM (291 μl, 2.65 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 24 h. Additional azide (1 eq) was added and the mixture, after being stirred for an additional 24 h, was concentrated. Treatment of the residue with 5% citric acid afforded a powder, which was purified by gel-filtration on Sephadex LH-20 as stated above, followed by precipitation twice from DMF with AcOEt; yield 1.80 g (60%), mp 168—170 °C, $[\alpha]_D^{25}$ +3.0° (*c* = 1.0, DMF), $Rf_1$ 0.37. *Anal.* Calcd for $C_{154}H_{238}N_{36}O_{42}S_4 \cdot 6H_2O$: C, 52.81; H, 7.20; N, 14.39. Found: C, 52.52; H, 7.04; N, 14.27.

**Z(OMe)–His–Ser(Bzl)–Asp–Gly–Thr–Phe–Thr–Ser–Glu–Leu–Ser–Arg(Mts)–Leu–Arg(Mts)–Asp–Ser(Bzl)–Ala–Arg(Mts)–Leu–Gln–Arg(Mts)–Leu–Leu–Gln–Gly–Leu–Val–$NH_2$ [Z(OMe)–(pS 1—27)–$NH_2$]**——A TFA-treat-

ed sample of Z(OMe)–(pS 7—27)–$NH_2$ (500 mg, 147 μmol) was dissolved in DMF (5 ml) containing $Et_3N$ (20.3 μl, 147 μmol). The azide [prepared from 411 mg (972 μmol) of Z(OMe)–(pS 1—6)–$NHNH_2$] in DMF (5 ml) and NMM (48.6 μl, 442 μmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 24 h, was concentrated. Treatment of the residue with 5% citric acid afforded a powder, which was purified by gel-filtration on Sephadex LH-20 as stated above, followed by precipitation from DMF with AcOEt; yield 520 mg (86%), mp 165—168 °C, $[\alpha]_D^{27}$ +8.9° ($c$=0.7, DMF), $Rf_1$ 0.30, $Rf_5$ 0.85. *Anal.* Calcd for $C_{189}H_{280}N_{44}O_{52}S_4 \cdot 4H_2O$: C, 54.03; H, 6.91; N, 14.67. Found: C, 53.97; H, 7.14; N, 14.75.

**H–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–$NH_2$ [H–(pS 1—27)–$NH_2$]**——The above protected heptacosapeptide amide (200 mg, 48 μmol) was treated with 1 M TFMSA–thioanisole in TFA (9.7 ml) in the presence of *m*-cresol (0.35 ml, 70 eq) in an ice-bath for 60 min, then dry ether was added. The resulting powder was collected by centrifugation, and dried over KOH pellets *in vacuo* for 30 min. This treatment was repeated twice more to ensure complete deprotection. The deprotected peptide thus obtained was dissolved in $H_2O$ (5 ml) and treated with Amberlite IR-400 (acetate form, approximately 2 g) for 30 min with stirring. The pH of the filtrate was adjusted to 8.0 with ice-chilled 2 N $NH_4OH$, then after 15 min, to 6.5 with 2 N AcOH. After lyophilization, the residual powder was dissolved in 3% AcOH (4 ml) and the solution was applied to a column of Sephadex G-25 (2.2 × 48 cm), which was eluted with the same solvent. Each fraction (3 ml) was examined by the conventional CBB G-250 method, and the UV absorption was measured at 240 nm. The fractions corresponding to the main peak (Fig. 6-A, tube Nos. 35—44) were combined and the solvent was removed by lyophilization to give a white fluffy powder; yield 101 mg (68%).

The above crude product was dissolved in pH 6.0, 0.01 M $KH_2PO_4$–NaOH buffer (4 ml) and the solution was applied to a column of SP-Sephadex C-25 (12 g, 2.6 × 25 cm), which was eluted first with the same buffer (9 ml) and then with a linear gradient formed by flowing pH 8.5, 0.1 M $KH_2PO_4$–NaOH buffer (500 ml) through a mixing flask containing the starting buffer (500 ml). Each fraction (9 ml) was examined by UV absorption measurement at 215 nm. The fractions corresponding to the main peak (Fig. 6-B, tube Nos. 60—100) were combined and the solvent was removed by lyophilization, then the product was desalted by gel-filtration on Sephadex G-25 in the same manner as described above, followed by lyophilization to give a white fluffy powder, yield 30.8 mg (30%).

Subsequent purification was performed by RP-HPLC on the Chemopak column. A part of the above purified sample (1 mg) was applied to the column, which was eluted with 0.1% TFA containing 32.5% acetonitrile under isocratic elution conditions at a flow rate of 1 ml/min. The eluate corresponding to the main peak (Fig. 7-A, determined by UV absorption measurement at 280 nm; retention time of 5.9 min) was collected. The rest of the sample (26 mg) was similarly purified and the solvent was removed by evaporation *in vacuo* at 30 °C or less. The residue was dissolved in 0.1 M NaOAc (2 ml) and the solution was desalted on Sephadex G-25 (2.2 × 48 cm), using 3% AcOH as an eluant. The desired fractions were collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 20.5 mg (76% in the HPLC purification step, 23% from the protected peptide), $[\alpha]_D^{28}$ −61.0° ($c$=0.4, 3% AcOH), $Rf_5$ 0.32, $Rf_7$ 0.24. The synthetic peptide exhibited a single peak on RP-HPLC (Fig. 7-B). Amino acid ratios in a 6 N HCl hydrolysate are listed in Table I. Amino acid ratios in LAP (purchased from Sigma Chemical Co., Lot. 79C-8110) digest (numbers in parentheses are theoretical values): His 0.93 (1), Ser 3.66 (4), Asp 1.89 (2), Gly 1.98 (2), Thr 1.91 (2), Phe 0.88 (1), Glu 1.06 (1), Leu 5.92 (6), Arg 3.99 (4), Ala 1.02 (1), Val 1.00 (1), Gln (2) was not determined (recovery of Val 74%). *Anal.* Calcd for $C_{130}H_{220}N_{44}O_{41} \cdot CH_3COOH \cdot 16H_2O$: C, 46.58; H, 7.58; N, 18.11. Found: C, 46.51; H, 7.28; N, 18.07.

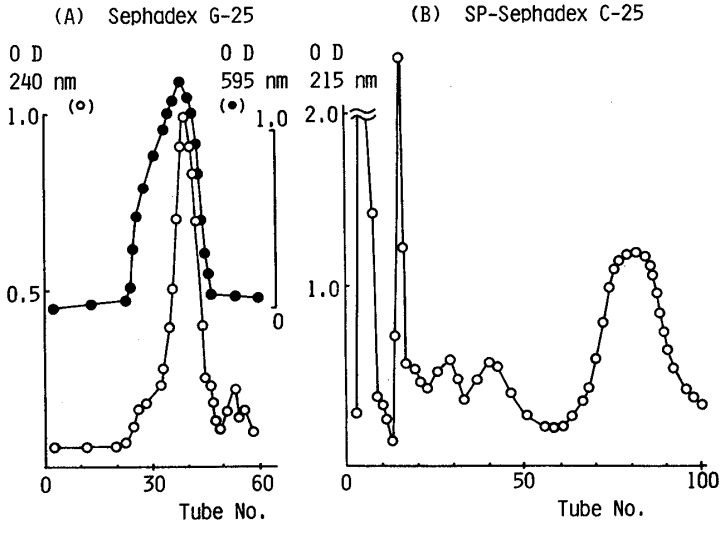


Fig. 6. Elution Profiles of Deprotected Porcine Secretin

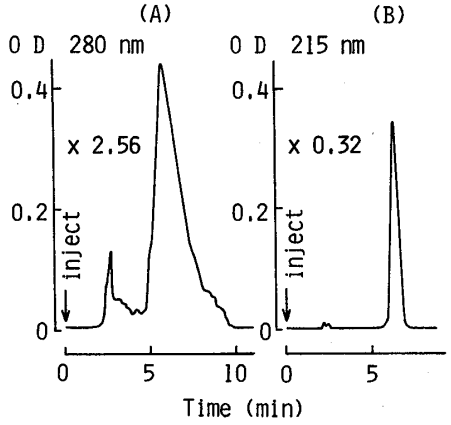


Fig. 7. HPLC of Synthetic Porcine Secretin

TABLE III. Physical Data for the Model Tetrapeptides

| | *Rf* | $[\alpha]_D^{28}$ | Formula | Analysis (%) Calcd (Found) | | | 6 N HCl hydrolysate (LAP digest) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | C | H | N | | | | |
| (I) | $Rf_5$ 0.21 | −17.5° ($c$=0.9) | $C_{15}H_{22}N_6O_8 \cdot H_2O$ | 41.66 (41.87 | 5.59 5.42 | 19.44 19.26) | His | 0.96 (1.00) | Ser | 0.91 (0.94) |
| | | | | | | | Asp | 1.00 (0.97) | Gly | 1.00 (1.00) |
| (II) | $Rf_5$ 0.21 | −10.0° ($c$=1.0) | $C_{15}H_{23}N_7O_7$ | 43.58 (43.81 | 5.61 5.60 | 23.72 23.77) | His | 0.95 (0.97) | Ser | 0.91 (0.93) |
| | | | | | | | Asp | 1.01 | Gly | 1.00 (1.00) |
| (III) | $Rf_5$ 0.25 | −0.1° ($c$=0.5) | $C_{16}H_{24}N_6O_8$ | 44.86 (44.55 | 5.65 5.85 | 19.62 19.36) | His | 1.00 (0.98) | Ser | 0.95 (0.96) |
| | | | | | | | Glu | 1.10 (1.00) | Gly | 1.00 (1.00) |
| (IV) | $Rf_1$ 0.20 | −37.7° ($c$=0.4) | $C_{15}H_{22}N_6O_7 \cdot H_2O$ | 43.26 (43.35 | 5.81 5.60 | 20.18 19.84) | His | 0.96 (1.00) | Ala | 1.00 (0.99) |
| | | | | | | | Asp | 1.06 (1.01) | Gly | 1.00 (1.00) |
| (V) | $Rf_3$ 0.67 | −25.6° ($c$=0.8) | $C_{15}H_{27}N_5O_8 \cdot 2H_2O$ | 40.81 (40.99 | 7.08 6.92 | 15.87 15.55) | Lys | 0.98 (1.00) | Ser | 0.91 (0.97) |
| | | | | | | | Asp | 1.01 (0.96) | Gly | 1.00 (1.00) |
| (VI) | $Rf_5$ 0.17 | −30.0° ($c$=0.5) | $C_{12}H_{20}N_4O_8 \cdot H_2O$ | 39.34 (39.26 | 6.05 6.35 | 15.30 15.50) | Ala | 1.01 (0.99) | Ser | 0.92 (0.98) |
| | | | | | | | Asp | 0.99 (0.95) | Gly | 1.00 (1.00) |

**2. Release of His from Synthetic Porcine Secretin**

A solution of H–(pS 1—27)–$NH_2$ (2.92 mg, 0.95 μmol) in $H_2O$ (1.0 ml, pH 5.0 adjusted with AcOH) and a solution of H–(pS 1—27)–$NH_2$ (3.15 mg, 1.03 μmol) in Tris–HCl buffer (1.0 ml, pH 7.73) were each incubated at 37 °C. An aliquot of each solution, after dilution with 0.02 N HCl, was tested on an amino acid analyzer. Release of His after 30 d: pH 5.0, 2.1%; pH 7.73, 3.6%.

**3. Syntheses of His–Ser–Asp–Gly and 5 Analogs**

**(I) Synthesis of His–Ser–Asp–Gly**——Z(OMe)–Asp(OBzl)–Gly–OBzl: This dipeptide ester was prepared by the Np method and purified by procedure A followed by recrystallization from MeOH with ether; yield 78%, mp 61—62 °C, $[\alpha]_D^{25}$ −1.8° ($c$=1.0, DMF), $Rf_6$ 0.34. *Anal.* Calcd for $C_{29}H_{30}N_2O_8$: C, 65.16; H, 5.66; N, 5.24. Found: C, 65.32; H, 5.74; N, 5.17.

Z(OMe)–His–Ser(Bzl)–Asp(OBzl)–Gly–OBzl: This tetrapeptide ester was prepared by the azide condensation of Z(OMe)–His–Ser(Bzl)–$NHNH_2$ with a TFA-treated sample of Z(OMe)–Asp(OBzl)–Gly–OBzl and purified by procedure A followed by recrystallization from MeOH with ether; yield 68%, mp 118—120 °C, $[\alpha]_D^{20}$ −8.6° ($c$=0.9, DMF), $Rf_1$ 0.43. *Anal.* Calcd for $C_{45}H_{48}N_6O_{11} \cdot H_2O$: C, 62.34; H, 5.81; N, 9.70. Found: C, 62.40; H, 5.88; N, 9.90.

H–His–Ser–Asp–Gly–OH (I): The above protected tetrapeptide (540 mg, 636 μmol) was treated with 1 M TFMSA–thioanisole in TFA (7.6 ml) in the presence of *m*-cresol (0.83 ml, 12 eq) in an ice-bath for 60 min, then dry ether was added. The resulting precipitate was dissolved in $H_2O$ (4 ml). The pH of the solution was adjusted to 8.0 with 2 N $NH_4OH$ and after 10 min in an ice-bath, to 6.0 with 2 N AcOH. The solution was applied to a column of Sephadex G-10 (2.2 × 45 cm), which was eluted with 3% AcOH. Individual fractions (4 ml) were checked by means of the ninhydrin color test on TLC and tube Nos. 31—34 were pooled. After lyophilization, a white fluffy powder was obtained; yield 111 mg (42%). Physical data are listed in Table III together with those of the other analogs.

**(II) Synthesis of His–Ser–Asn–Gly**——Z(OMe)–Asn–Gly–OBzl: The title compound was prepared by the Np active ester method in DMSO. Purification was performed by procedure B followed by recrystallization from MeOH with ether; yield 84%, mp 165—166 °C, $[\alpha]_D^{28}$ −1.4° ($c$=1.0, DMF), $Rf_1$ 0.34. *Anal.* Calcd for $C_{22}H_{25}N_3O_7$: C, 59.58; H, 5.68; N, 9.48. Found: C, 59.58; H, 5.77; N, 9.56.

Z(OMe)–His–Ser(Bzl)–Asn–Gly–OBzl: The title compound was prepared by the usual azide method from Z(OMe)–His–Ser(Bzl)–$NHNH_2$ and a TFA-treated sample of Z(OMe)–Asn–Gly–OBzl. Purification was performed

by procedure B followed by recrystallization from MeOH with ether; yeild 85%, mp 184—186 °C, $[\alpha]_D^{30}$ −3.0° ($c$=1.0, DMF), $Rf_1$ 0.41. *Anal.* Calcd for $C_{38}H_{43}N_7O_{10}$: C, 60.23; H, 5.72; N, 12.94. Found: C, 60.52; H, 5.49; N, 13.01.

H–His–Ser–Asn–Gly–OH (II): The deprotection of the above protected tetrapeptide and subsequent purification was performed in the same manner as described for compound (I); yield 65%.

**(III) Synthesis of His–Ser–Glu–Gly**——Z(OMe)–His–Ser(Bzl)–Glu(OBzl)–Gly–OBzl: Z(OMe)–Glu(OBzl)–Gly–OBzl was prepared by the Np active ester method. The oily product, after being purified by procedure A then treated with TFA, was condensed with Z(OMe)–His–Ser(Bzl)–$NHNH_2$ by the azide method. The product was purified by procedure A and recrystallized from MeOH with ether; yield 74%, mp 118—120 °C, $[\alpha]_D^{25}$ −1.0° ($c$=1.0, DMF), $Rf_3$ 0.74. *Anal.* Calcd for $C_{46}H_{50}N_6O_{11}$: C, 64.02; H, 5.84; N, 9.74. Found: C, 63.88; H, 5.96; N, 9.72.

H–His–Ser–Glu–Gly–OH (III): The above protected tetrapeptide was deprotected and purified in the same manner as described for compound (I); yield 67%.

**(IV) Synthesis of His–Ala–Asp–Gly**——Z(OMe)–His–Ala–Asp(OBzl)–Gly–OBzl: The title compound was prepared by the usual azide method from Z(OMe)–His–Ala–$NHNH_2$[38] and a TFA-treated sample of Z(OMe)–Asp(OBzl)–Gly–OBzl, and purified by procedure B followed by recrystallization from MeOH with ether; yield 70%, mp 127—129 °C, $[\alpha]_D^{25}$ −19.8° ($c$=0.6, DMF), $Rf_1$ 0.55. *Anal.* Calcd for $C_{38}H_{42}N_6O_{10}$: C, 61.44; H, 5.70; N, 11.32. Found: C, 61.39; H, 5.61; N, 11.51.

H–His–Ala–Asp–Gly–OH (IV): The above protected tetrapeptide was deprotected and purified in the same manner as described for compound (I); yield 57%.

**(V) Synthesis of Lys–Ser–Asp–Gly**——Z–Lys(Z)–Ser–OMe: The title compound was prepared by a mixed anhydride method and purified by procedure A followed by recrystallization from MeOH with ether; yield 81%, mp 90—92 °C, $[\alpha]_D^{25}$ −2.0° ($c$=1.0, DMF), $Rf_3$ 0.80. *Anal.* Calcd for $C_{26}H_{33}N_3O_8$: C, 60.57; H, 6.45; N, 8.15. Found: C, 60.83; H, 6.49; N, 7.98.

Z-Lys(Z)–Ser–$NHNH_2$: The above methyl ester was converted to its hydrazide by the usual hydrazine treatment and purified by reprecipitation from DMF with MeOH; yield 84%, mp 147—148 °C, $[\alpha]_D^{28}$ −11.2° ($c$=1.0, DMF), $Rf_1$ 0.67. *Anal.* Calcd for $C_{25}H_{33}N_5O_7$: C, 58.24; H, 6.45; N, 13.59. Found: C, 58.40; H, 6.42; N, 13.63.

Z–Lys(Z)–Ser–Asp(OBzl)–Gly–OBzl: The title compound was prepared by the usual azide condensation of Z–Lys(Z)–Ser–$NHNH_2$ and a TFA-treated sample of Z(OMe)–Asp(OBzl)–Gly–OBzl, and purified by procedure B followed by recrystallization from MeOH with ether; yield 50%, mp 147—150 °C, $[\alpha]_D^{27}$ −5.3° ($c$=0.7, DMF), $Rf_3$ 0.67. *Anal.* Calcd for $C_{45}H_{51}N_5O_{12}$: C, 63.29; H, 6.02; N, 8.20. Found: C, 62.96; H, 6.22; N, 8.15.

H–Lys–Ser–Asp–Gly–OH (V): The above protected tetrapeptide in MeOH was hydrogenated over a Pd catalyst for 4 h. The solution was filtered, the filtrate was concentrated and the residue was purified by gel-filtration on Sephadex G-10 using 3% AcOH as an eluant; yield 38%.

**(VI) Synthesis of Ala–Ser–Asp–Gly**——Z–Ala–Ser–OMe: The title compound was prepared by a mixed anhydride method and purified by procedure A followed by recrystallization from MeOH with petroleum ether; yield 69%, mp 145—147 °C, $[\alpha]_D^{27}$ +3.3° ($c$=0.3, DMF), $Rf_1$ 0.80. *Anal.* Calcd for $C_{15}H_{20}N_2O_6$: C, 55.55; H, 6.22; N, 8.64. Found: C, 55.80; H, 6.24; N, 8.77.

Z–Ala–Ser–$NHNH_2$: The above methyl ester was converted to its hydrazide by the usual hydrazine treatment and reprecipitated from MeOH with $H_2O$; yield 77%, mp 227—229 °C, $[\alpha]_D^{27}$ +9.6° ($c$=1.0, DMF), $Rf_1$ 0.72. *Anal.* Calcd for $C_{14}H_{20}N_4O_5$: C, 51.84; H, 6.22; N, 17.28. Found: C, 51.88; H, 6.23; N, 17.06.

Z–Ala–Ser–Asp(OBzl)–Gly–OBzl: The title compound was prepared by the usual azide condensation of Z–Ala–Ser–$NHNH_2$ and a TFA-treated sample of Z(OMe)–Asp(OBzl)–Gly–OBzl and purified by procedure A followed by recrystallization from MeOH with ether; yield 42%, mp 150—151 °C, $[\alpha]_D^{28}$ −7.0° ($c$=1.0, DMF), $Rf_3$ 0.84. *Anal.* Calcd for $C_{34}H_{38}N_4O_{10}$: C, 61.62; H, 5.78; N, 8.64. Found: C, 61.52; H, 5.72; N, 8.31.

H–Ala–Ser–Asp–Gly–OH (VI): The above protected tetrapeptide in MeOH was hydrogenated over a Pd catalyst and purified in the same manner as described for compound (V); yield 75%.

**4. Incubation of His–Ser–Asp–Gly and the 5 Analogs in Aqueous Solution**

Solutions of a test sample (concentration: 6.20 μmol/ml) in $H_2O$ (pH 5.0 adjusted with AcOH) and in Tris–HCl buffer (pH 7.73) were incubated at 37 °C. Aliquots of each solution, after dilution with 0.02 N HCl, were tested periodically on an amino acid analyzer. The results are shown in Fig. 4.

**5. HPLC Examination of Incubated Synthetic Porcine Secretin**

A secretin solution incubated at pH 5.0 (as described in section **2**) was examined by RP-HPLC on the Chemopak column with linear gradient elution from 0.1% TFA containing 15% acetonitrile to 0.1% TFA containing 90% acetonitrile during 75 min at a flow rate of 1 ml/min. The elution pattern is shown in Fig. 5. Eluates corresponding to 6 peaks were separately collected, the solvent was evaporated off, and the residues were each hydrolyzed with 6 N HCl. These results are listed in Table II.

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**References and Notes**

1) Amino acids, peptides and their derivatives in this paper are of the L-configuration. Abbreviations used are

those recommended by the I.U.P.A.C.-I.U.B. Commission on Biochemical Nomenclature: *J. Biol. Chem.*, **247**, 977 (1972). Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Boc = *tert*-butyloxycarbonyl, Bzl = benzyl, Bu$^t$ = *tert*-butyl, Mts = mesitylenesulfonyl, Np = *p*-nitrophenyl, Su = *N*-hydroxysuccinimidyl, DNp = 2,4-dinitrophenyl, DCC = *N*, *N'*-dicyclohexylcarbodiimide, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, $Et_3N$ = triethylamine, NMM = *N*-methylmorpholine, DMF = dimethylformamide, DMSO = dimethylsulfoxide, MeOH = methanol, EtOH = ethanol, AcOH = acetic acid, AcOEt = ethyl acetate, CHA = cyclohexyl amine.


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