Cholecystokinin–Pancreozymin. I. The Synthesis of Peptides Corresponding to the N-Terminal Sequence[1]

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The preparation of a protected octapeptide, bis-*tert*-butyloxycarbonyl-L-lysyl-L-alanyl-L-prolyl-L-serylglycyl-L-arginyl-L-valyl-L-serine hydrazide (XVIII), is described. The synthesis of a hexapeptide, L-lysyl-L-alanyl-L-prolyl-L-serylglycyl-L-arginine (X), identical with the N-terminal tryptic peptide of cholecystokinin–pancreozymin, is also reported.

The sequence of the 33 amino acids which constitute the single chain of the intestinal hormone cholecystokinin–pancreozymin (CCK) has been revealed by the investigations of Jorpes and Mutt.[2,3] A dodecapeptide identical with the C-terminal tryptic peptide of the hormone has been synthesized by Ondetti and his coworkers,[4] as well as several analogs of that sequence.[5] Because of the problems anticipated in working in a stepwise manner[6] with peptides containing an L-tyrosine-*O*-sulfate residue, a fragment condensation strategy seemed to be advisable for the total synthesis of the hormone. The preparation of a protected octapeptide, bis-*tert*-butyloxycarbonyl-L-lysyl-L-alanyl-L-prolyl-L-serylglycyl-L-arginyl-L-valyl-L-serine hydrazide (XVIII), which corresponds to the 1–8 sequence of the hormone, is described. This derivative is expected to be applicable in the synthesis of the 1–16 sequence of the hormone, and eventually of the whole molecule or its analogs. This paper deals in part with the synthesis of a free hexapeptide, L-lysyl-L-alanyl-L-prolyl-L-serylglycyl-L-arginine, corresponding in sequence to the N-terminal tryptic peptide. The syntheses of these two peptides are represented in Charts I, II, and III.

The intermediate common in both syntheses, the pentapeptide derivative bis-*tert*-butyloxycarbonyl-L-lysyl-L-alanyl-L-prolyl-*O*-benzyl-L-serylglycine ethyl ester (VII), was prepared in a stepwise manner. *N-tert*-Butyloxycarbonyl-*O*-benzyl-L-serine and glycine ethyl ester hydrochloride were coupled with dicyclohexylcarbodiimide[7] in the presence of diisopropylethylamine.[8] The dipeptide I was deprotected with trifluoroacetic acid, and acylated with *N-tert*-butyloxycarbonyl-L-proline *p*-nitrophenyl ester.[9] The peptide chain was elongated by the similar application of active esters of *N-tert*-butyloxycarbonyl-L-alanine[10] and bis-*tert*-butyloxycarbonyl-L-lysine.[11]

For the synthesis of the N-terminal hexapeptide X, the pentapeptide ester VII was converted to the corresponding hydrazide VIII and, after treatment with nitrous acid, was coupled to L-nitroarginine benzyl ester.[12] The protected hexapeptide IX was deblocked in two stages (Chart I). The product X was shown to be chromatographically and electrophoretically indistinguishable from the peptide isolated from the tryptic digest of cholecystokinin–pancreozymin.[13]

Initially, synthesis of protected octapeptide hydrazide XVIII was attempted by coupling the pentapeptide azide prepared from VIII to L-nitroarginyl-L-valyl-*O*-benzyl-L-serine *p*-nitrobenzyloxycarbonyl hydrazide. Hydrogenation of the product gave a multicomponent mixture. A more successful approach to the synthesis of XVIII (Chart II) started with the coupling of *N*-benzyloxycarbonyl-L-valine to serine methyl ester with dicyclohexylcarbodiimide.[7] (Woodwards Reagent K[14] proved less satisfactory in this coupling reaction.) The protected dipeptide XI was hydrogenated in the presence of 1 equiv of hydrochloric acid, and the deprotected dipeptide XII was acylated with *N*-benzyloxycarbonyl-L-nitroarginine pentachlorophenyl ester.[15] The tripeptide XIII was readily purified by recrystallization and was hydrogenated, in the presence of 5 equiv of hydrochloric acid, to simultaneously remove the *N*-benzyloxycarbonyl and nitro groups, giving the tripeptide XIV as a dihydrochloride. The pentapeptide hydrazide XVI was best prepared by hydrogenation of peptide VII followed by treatment of the product with hydrazine, rather than by directly hydrogenating the pentapeptide hydrazide VIII. The coupling reaction of the azide formed from a slight excess of the hydrazide XVI with the tripeptide dihydrochloride XIV proceeded well, and the product, the octapeptide XVII, could be purified by chromatography on carboxymethylcellulose. This octapeptide ester was rapidly and smoothly converted into the desired hydrazide XVIII on treatment with excess hydrazine in methanol for 24 hr. Since the suitability of XVIII as an intermediate in the synthesis of CCK by fragment condensation remains somewhat uncertain, an alternative route, involving an active ester of the N-terminal pentapeptide, was also explored. The synthesis of this active ester, bis-*tert*-butyloxycarbonyl-L-lysyl-L-alanyl-L-prolyl-L-serylglycine pentachlorophenyl ester (XXVII), is summarized in Chart III, which also shows the coupling of XXVII to XIV, yielding XVIII.

(1) This work was supported by a grant from the U. S. Public Health Service (NIH 5-RO1-AM12473).

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CHART I

SYNTHESIS OF THE HEXAPEPTIDE CCK_{1-6}

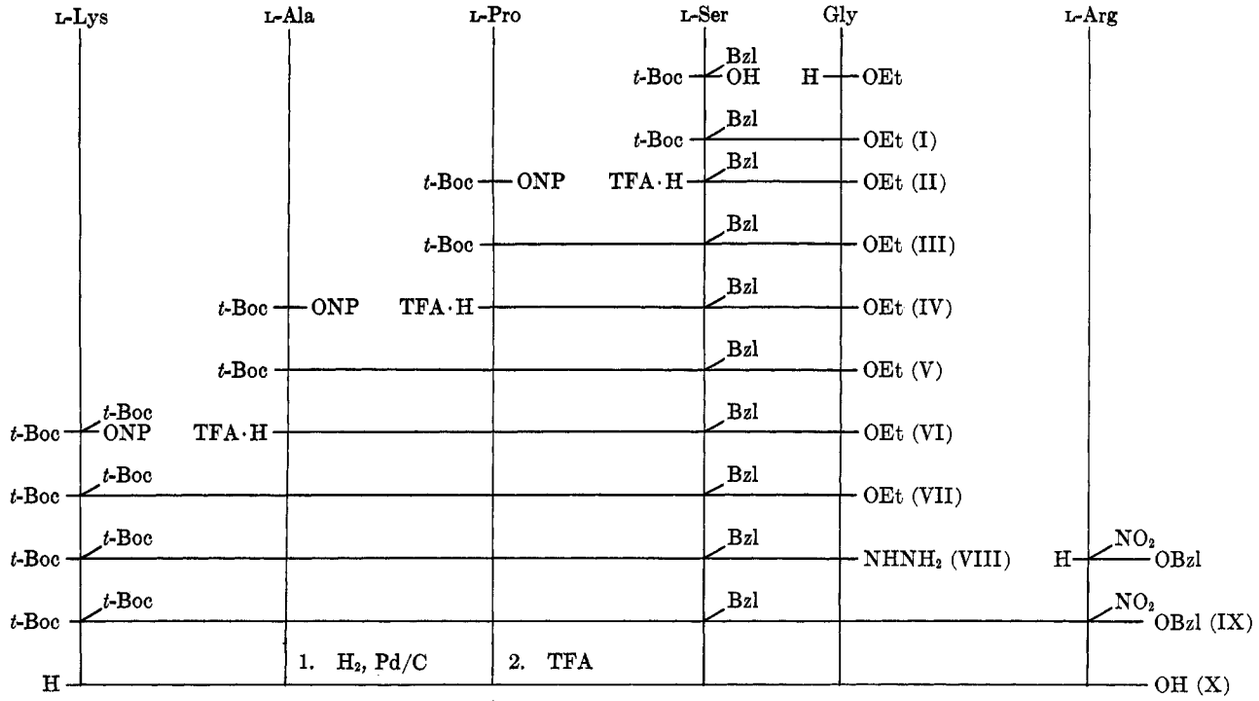
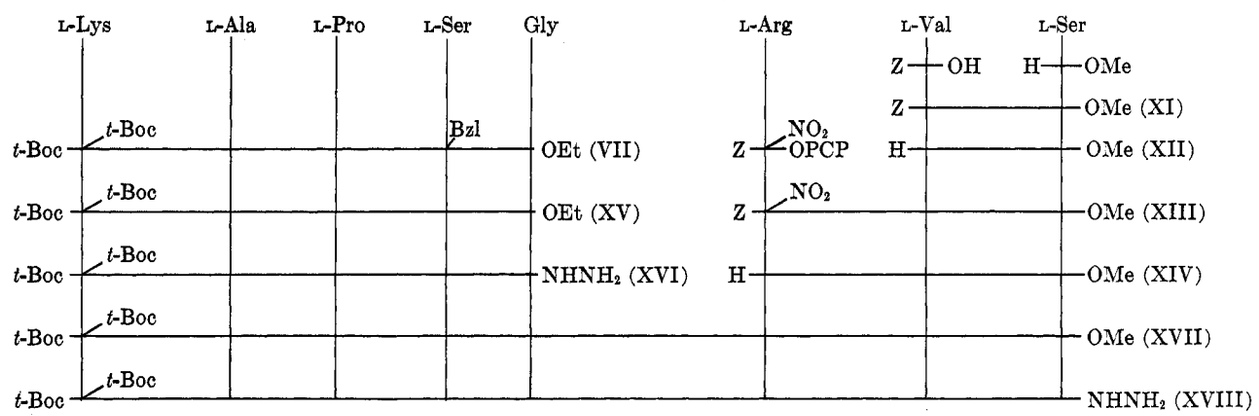


CHART II

AZIDE SYNTHESIS OF THE PROTECTED OCTAPEPTIDE (XVIII) HYDRAZIDE CORRESPONDING TO CCK_{1-8}



Experimental Section

Capillary melting points are reported uncorrected. For thin layer chromatography, the protected peptides were revealed by *tert*-butyl hypochlorite–KI–starch reagents,[16] or by the method of charring developed by Ziminski and Borowski.[17] The following solvent systems were used for development: A, *n*-BuOH–AcOH–H_2O (4:1:1); B, *n*-BuOH–AcOH–H_2O (3:1:1); C, *n*-BuOH–pyridine–AcOH–H_2O (30:20:6:24); D, EtOAc–pyridine–AcOH–H_2O (60:20:6:11); E, $CHCl_3$–MeOH (9:1); F, *n*-BuOH–AcOH–H_2O (4:1:5); G, *n*-PrOH–H_2O (7:3).

For amino acid analysis, samples were hydrolyzed with constant-boiling HCl in evacuated, sealed ampoules at 110° for 16 hr, and analyzed by the Spackman–Stein–Moore method[18] on a Beckman Spinco 120C amino acid analyzer.

***N*-*tert*-Butyloxycarbonyl-*O*-benzyl-L-serylglycine Ethyl Ester (I).**—Glycine ethyl ester hydrochloride (2.8 g, 20 mmol), diisopropylethylamine[8] (2.60 g, 20 mmol), and *tert*-butyloxycarbonyl-*O*-benzyl-L-serine (2.96 g, 10 mmol) were dissolved in $CHCl_3$ (30 ml). The solution was stirred and cooled to 0°, and dicyclohexylcarbodiimide (DCC, 2.5 g, 12 mmol) was added. After stirring overnight at room temperature, the mixture was filtered and the filtrate was evaporated. The oily residue was dissolved in EtOAc, and a few drops of AcOH were added. After 1 hr, the precipitated *N*,*N*′-dicyclohexylurea (DCU) was removed by filtration and the filtrate was washed with cold 10% citric acid solution, H_2O, 5% $NaHCO_3$ solution, and H_2O, dried over $MgSO_4$, and evaporated *in vacuo*. The sirupy residue was dissolved in ether, from which crystals of the dipeptide appeared on standing. Recrystallization from ether gave 3.3 g (86%): mp 87–88°; $[\alpha]^{24}D$ +24.5° (*c* 3, DMF); tlc R_f A 0.70, R_f C 0.69; amino acid analysis, Ser, 1.0, Gly, 1.1.

Anal. Calcd for $C_{19}H_{25}N_2O_6$ (380.4): C, 60.0; H, 7.4; N, 7.4. Found: C, 60.2; H, 7.5; N, 7.3.

***N*-*tert*-Butyloxycarbonyl-L-prolyl-*O*-benzyl-L-serylglycine Ethyl Ester (III).**—The protected dipeptide I (5 g, 13 mmol) was dissolved in trifluoroacetic acid (TFA, 50 ml). After 30 min at 15°, the TFA was evaporated *in vacuo* and the residue was triturated with ether, filtered, washed with ether, and dried to give 4.6 g (91%): mp 116–117°; tlc R_f A 0.55, R_f C 0.62.

The dipeptide trifluoroacetate II (4.3 g, 11 mmol), *tert*-butyloxycarbonyl-L-proline *p*-nitrophenyl ester[9] (4.0 g, 12 mmol), and triethylamine (TEA, 1.1 g, 11 mmol) were dissolved in CH_3CN (25 ml), and the solution was stirred overnight. The

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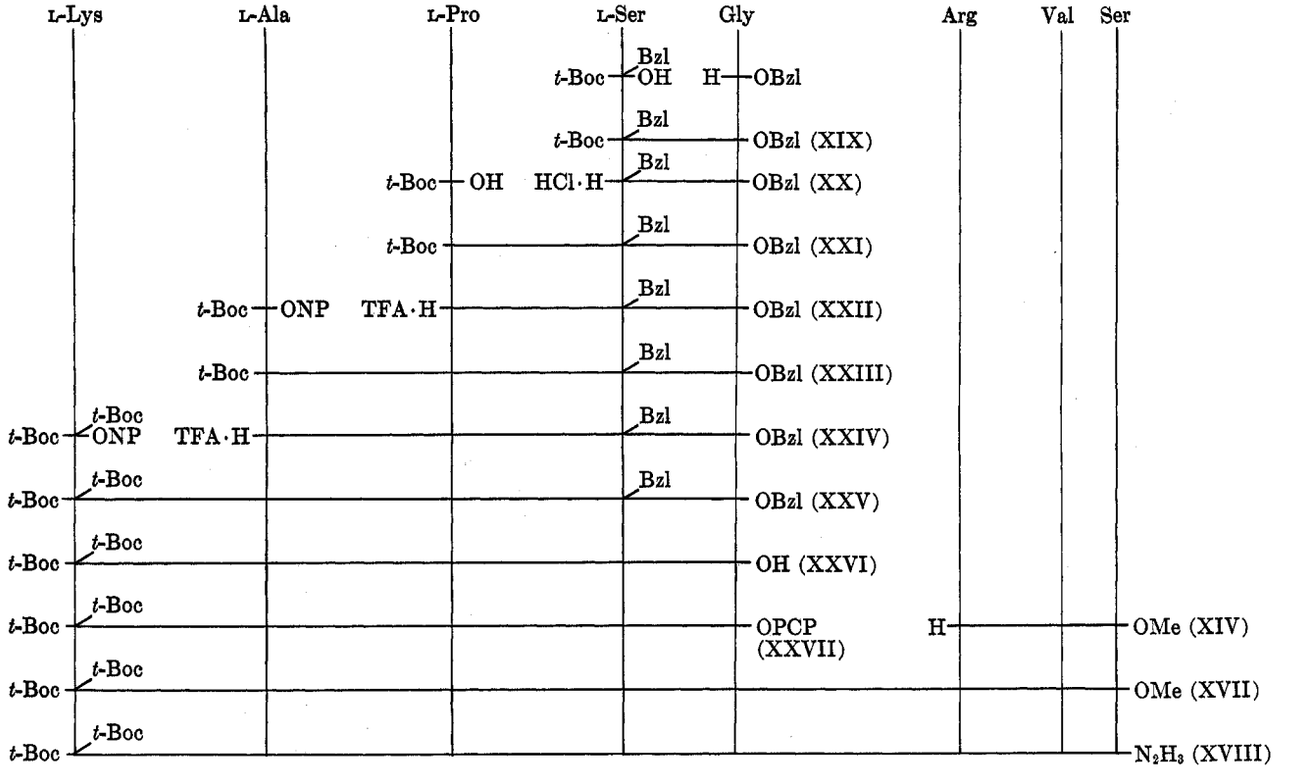
CHART III

ACTIVE ESTER SYNTHESIS OF XVIII



solvent was replaced with EtOAc, and the solution was washed with cold 10% citric acid solution, H_2O, and 0.75 *M* NH_4OH. The organic phase was dried over $MgSO_4$ and then evaporated to a sirupy residue which was dissolved in ether. On standing, crystals (1.3 g) separated. The mother liquors were treated with unsymmetrical dimethylethylenediamine[19] (0.88 g, 10 mmol) and, after 6 hr, the solution was washed with citric acid solution and dilute NH_4OH. On evaporation, an additional amount (2.5 g) of the pure tripeptide was obtained, giving a total yield of 3.8 g (60%): mp 109–110°; $[\alpha]^{24}D$ −9° (*c* 2.25, DMF); tlc R_f A 0.70, R_f C 0.69; amino acid analysis, Pro, 1.0, Ser, 0.8, Gly, 0.9.

Anal. Calcd for $C_{24}H_{35}N_3O_7$ (477.6): C, 60.4; H, 7.4; N, 8.8. Found: C, 60.5; H, 7.5; N, 9.0.

***N*-tert-Butyloxycarbonyl-L-alanyl-L-prolyl-*O*-benzyl-L-serylglycine Ethyl Ester (V).**—The tripeptide III (3.8 g, 8 mmol) was dissolved in TFA (40 ml). After 30 min at 20°, the TFA was evaporated *in vacuo*, and the residue was triturated with ether, washed with ether, and dried to give IV: 3.5 g (89%); mp 162–163°; paper chromatography R_f F 0.71, R_f C 0.77; tlc R_f A 0.44, R_f C 0.60.

The tripeptide trifluoroacetate IV (3.45 g, 7 mmol), TEA (0.7 g, 7 mmol), and *tert*-butyloxycarbonyl-L-alanine *p*-nitrophenyl ester[10] (2.5 g, 8 mmol) were dissolved in CH_3CN (30 ml). After the solution had been stirred overnight, the excess of *p*-nitrophenyl ester was decomposed with unsymmetrical dimethylethylenediamine.[19] The mixture was worked up as described for compound III to give 2.86 g (75%): mp 132–133°; $[\alpha]^{24}D$ −23.5° (*c* 1.5, DMF); tlc R_f A 0.69, R_f B 0.69; amino acid analysis, Ala, 1.0, Pro, 1.0, Ser, 1.0, Gly, 1.1.

Anal. Calcd for $C_{27}H_{40}N_4O_8$ (548.6): C, 59.1; H, 7.3; N, 10.2. Found: C, 59.1; H, 7.4; N, 10.2.

N^{α},N^{ϵ}-Bis-*tert*-butyloxycarbonyl-L-lysyl-L-alanyl-L-prolyl-*O*-benzyl-L-serylglycine Ethyl Ester (VII).—The *tert*-butyloxycarbonyl group was removed from compound V (2.75 g, 5.0 mmol) as described above to give the trifluoroacetate VI (2.5 g, 90%): paper chromatography R_f A 0.62, R_f C 0.67; tlc R_f A 0.5, R_f C 0.5.

The tetrapeptide trifluoroacetate VI (2.5 g, 4.5 mmol) was suspended in CH_3CN (30 ml), and TEA (0.45 g, 4.5 mmol) and bis-*tert*-butyloxycarbonyl-L-lysine *p*-nitrophenyl ester[11] (2.55 g, 5.5 mmol) were added. The mixture was stirred overnight at room temperature. The excess of *p*-nitrophenyl ester was decomposed and the reaction mixture was worked up as described for the preparation of compound V. The sirupy crude product solidified on addition of ether to give 2.9 g (83%). For analysis, a sample was crystallized from MeOH–ether: mp 131–132°; $[\alpha]^{24}D$ −30° (*c* 1.67, DMF); tlc R_f A 0.70, R_f C 0.70; amino acid analysis, Lys, 1.0, Ala, 1.0, Pro, 1.2, Ser, 0.9, Gly, 0.9.

Anal. Calcd for $C_{38}H_{60}N_6O_{11}$ (776.9): C, 58.7; H, 7.8; N, 10.8. Found: C, 58.8; H, 7.90; N, 10.9.

N^{α},N^{ϵ}-Bis-*tert*-butyloxycarbonyl-L-lysyl-L-alanyl-L-prolyl-*O*-benzyl-L-serylglycine Hydrazide (VIII).—The protected pentapeptide ethyl ester VII (4.0 g, 5.15 mmol) was dissolved in MeOH (80 ml) and treated with hydrazine (1.8 g), and the solution was stored at room temperature for 4 days. The solvent was evaporated and the residue was dried *in vacuo* over concentrated H_2SO_4. The product was obtained from MeOH–ether as an amorphous solid (3.6 g, 80%): mp 100–105°; $[\alpha]^{24}D$ −66° (*c* 1.5, MeOH); tlc R_f A 0.63, R_f D 0.56.

Anal. Calcd for $C_{36}H_{58}N_8O_{10}$ (762.9): C, 56.7; H, 7.7; N, 14.7. Found: C, 56.8; H, 7.8; N, 14.5.

L-Lysyl-L-alanyl-L-prolyl-L-serylglycyl-L-arginine.—The protected pentapeptide hydrazide VIII (0.76 g, 1 mmol) was dissolved in DMF (6 ml) and the stirred solution was cooled to −20°. Concentrated HCl (0.5 ml, *ca.* 5.0 mmol) and then 2 *M* $NaNO_2$ solution (0.75 ml, 1.5 mmol) were slowly added. After 5 min at −15°, the temperature was lowered to −25°, and TEA (0.7 ml, 5 mmol) and nitro-L-arginine benzyl ester[12] (0.6 g, 2 mmol) were added. The mixture was kept at 4° for 3 days. The solvent was evaporated *in vacuo*, and the residue was partitioned between EtOAc and H_2O. The EtOAc layer was washed with cold 10% citric acid solution and 5% $NaHCO_3$, then dried over $MgSO_4$ and evaporated. The sirupy residue solidified under ether and the product, N^{α},N^{ϵ}-bis-*tert*-butyloxycarbonyl-L-lysyl-L-alanyl-L-prolyl-*O*-benzyl-L-serylglycylnitro-L-arginine benzyl ester, was filtered, washed with excess ether, and dried to give 0.9 g (85%): mp 94–95°; tlc R_f A 0.6; amino acid analysis, Orn + Lys, 1.45, Ala, 1.0, Pro, 1.0, Ser, 0.95, Gly, 1.0, Arg, 0.70 (nitroarginine content = 1.0 residue per mole, determined by uv absorption at 270 nm).

The protected hexapeptide (1.2 g, 1.14 mmol) was hydrogenated for 4 days in the presence of 10% Pd on charcoal catalyst (0.3 g) in a mixture of EtOH (20 ml) and AcOH (2 ml).

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The catalyst was removed and the solution was evaporated to dryness. The residue was dried *in vacuo* and washed with ether and EtOAc to give 0.9 g (88%) of N^{α},N^{ϵ}-bis-*tert*-butyloxycarbonyl-L-lysyl-L-alanyl-L-prolyl-L-serylglycyl-L-arginine: mp 191–193° (hygroscopic); $[\alpha]^{24}D$ −20° (*c* 1, DMF); tlc R_f A 0.35.

Anal. Calcd for $C_{55}H_{62}N_{10}O_{12}\cdot CH_3CO_2H\cdot 2H_2O$ (895.0): C, 48.8; H, 7.5; N, 15.4. Found: C, 48.3; H, 7.3; N, 15.8.

This partially blocked peptide (0.5 g, 0.56 mmol) was treated with TFA (10 ml) at room temperature for 30 min. The TFA was evaporated and the residue, after trituration under ether, was precipitated from MeOH with EtOAc to give 0.4 g: mp 170–175° dec; $[\alpha]^{24}D$ −21° (*c* 1, DMF); on paper R_f F 0.08, R_f C 0.05; does not move on tlc.

The hexapeptide ditrifluoroacetate (200 mg) was chromatographed on a column of Amberlite IRC-50 (2.5 × 30 cm) with ammonium acetate buffer, pH 6.5, of successively increasing ammonium ion concentration. Fractions of 7 ml each were collected and tested with Sakaguchi reagent. Tubes 240–275, eluted with 1.0 *M* buffer, contained X in electrophoretically homogeneous form. After lyophilization, 100 mg of pure peptide was obtained: amino acid analysis, Lys, 1.15, Arg, 0.95, Ser, 0.95, Pro, 1.0, Gly, 1.0, Ala, 1.0.

***N*-Benzyloxycarbonyl-L-valyl-L-serine Methyl Ester (XI).**—*N*-Benzyloxycarbonyl-L-valine (5.0 g, 20 mmol) and L-serine methyl ester hydrochloride (3.1 g, 20 mmol) were dissolved in CH_2Cl_2 (100 ml), and the solution was cooled to −4°. TEA (2.80 ml, 20 mmol) and DCC were added, in that order, and the mixture was stirred overnight. The precipitate was collected by filtration and washed with CH_2Cl_2 (100 ml). The combined filtrates were washed with 0.5 *M* HCl (3 × 70 ml), 1.0 *M* $NaHCO_3$ solution (3 × 70 ml), and H_2O (3 × 70 ml). On evaporation of the organic phase, a crystalline residue was obtained which was extracted with acetone (2 × 100 ml). The residue, mp 164–165°, was recrystallized from warm EtOH, mp 166–167°. The acetone washes were evaporated, and the crystalline residue was extracted several times with ether and then recrystallized from warm EtOH, mp 165–166°. A total of 3.5 g (50%) of product was thus obtained: $[\alpha]^{24}D$ −19° (*c* 1, methanol); tlc R_f B 0.90, R_f E 0.84.

Anal. Calcd for $C_{17}H_{24}N_2O_6$ (352.4): C, 57.9; H, 6.9; N, 8.0. Found: C, 58.2; H, 6.9; N, 8.1.

***N*-Benzyloxycarbonyl-L-nitroarginyl-L-valyl-L-serine Methyl Ester (XIII).**—The protected dipeptide XI (2.37 g, 6.75 mmol) was dissolved in a mixture of MeOH (120 ml) and 1 *M* HCl (7.3 ml), and the solution was hydrogenated for 4 hr in the presence of 10% Pd on charcoal catalyst (1.0 g). The solution was filtered, and the filtrate was evaporated to dryness at room temperature. The crystalline hygroscopic residue was dried overnight *in vacuo* to give XII, 1.7 g (100%), R_f B 0.55. It was dissolved in DMF (30 ml), and TEA (0.95 ml, 6.75 mmol) and *N*-benzyloxycarbonyl-L-nitroarginine pentachlorophenyl ester[15] (4.08 g, 6.75 mmol) were added, in that order, to the stirred solution. After 1 day, the solution was evaporated to dryness and the residue was triturated under an EtOAc (30 ml) and H_2O (20 ml) mixture. The white crystalline material (2.0 g, mp 184–186°) was filtered and washed with EtOAc and H_2O. A further crop of material (0.6 g) was obtained on concentration of the mother liquors. The two crops were combined and recrystallized from EtOH to give 2.24 g (60%): mp 185–187°; $[\alpha]^{24}D$ +3° (*c* 1, DMF); tlc R_f D 0.85, R_f B 0.9; amino acid analysis, Val, 1.0, Ser, 0.9, (NO_2) Arg (from uv absorption at 270 nm), 1.0.

Anal. Calcd for $C_{23}H_{35}N_7O_9$ (553.6): C, 49.9; H, 6.4; N, 17.7. Calcd for $C_{23}H_{35}N_7O_9\cdot H_2O$ (571.6): C, 48.3; H, 6.5; N, 17.2. Found: C, 47.9; H, 6.6; N, 17.1.

N^{α},N^{ϵ}-Bis-*tert*-butyloxycarbonyl-L-lysyl-L-alanyl-L-prolyl-L-serylglycine Hydrazide (XVI).—The protected pentapeptide ethyl ester VII (1.35 g, 1.74 mmol) was dissolved in a mixture of EtOH (50 ml) and AcOH (4 ml). The solution was hydrogenated for 2 days in the presence of a 10% Pd on charcoal catalyst (0.5 g). The catalyst was removed, the solution was evaporated, and the residue was dried *in vacuo*. On trituration under ether, an amorphous hygroscopic solid was obtained: 1.2 g (*ca.* 100%); mp 130° (sintering at 90°); $[\alpha]^{24}D$ −45° (*c* 1.2, DMF); tlc R_f A 0.65; the nmr spectrum showed the absence of aromatic protons.

The partially protected pentapeptide ester XV (2.29 g, 3.34 mmol) was dissolved in MeOH (50 ml) and treated with hydrazine (1 g). After 2 days, the solution was evaporated to dryness and the oily residue was dried over concentrated H_2SO_4 *in vacuo*. The glassy product was dissolved in EtOAc and precipitated by the addition of ether. The hygroscopic amorphous product (2.25 g, 98%) was collected: mp 130° (sintering at 90°); $[\alpha]^{24}D$ −38° (*c* 1.1, DMF); tlc R_f A 0.54, R_f C 0.66, R_f D 0.65.

N^{α},N^{ϵ}-Bis-*tert*-butyloxycarbonyl-L-lysyl-L-alanyl-L-prolyl-L-serylglycyl-L-arginyl-L-valyl-L-serine Methyl Ester Acetate (XVII). Azide Method.—Compound XIII (2.0 g, 3.62 mmol) was dissolved in MeOH (200 ml) and 1 *M* HCl (20 ml), and hydrogenated for 40 hr in the presence of 10% Pd on charcoal catalyst (1.0 g). The solution was filtered and evaporated to dryness, and the residue was lyophilized from H_2O. The glasslike product amounted to 1.6 g (100%); tlc R_f B, D 0.0; on paper chromatograms, R_f C 0.65. The product XIV was used as such in the preparation of octapeptide XVII.

The pentapeptide hydrazide XVI (2.7 g, 4 mmol) was dissolved in DMF (40 ml), and the stirred solution was cooled to −25°. A solution of 4.8 *M* HCl in dioxane (4 ml) was slowly added, followed by isoamyl nitrite (0.655 ml, 4.8 mmol). After 15 min at −25°, the solution was cooled to −50° and TEA (2.69 ml) was added, followed by a precooled solution of the tripeptide dihydrochloride XIV (1.6 g, 3.62 mmol) and TEA (0.51 ml, 3.62 mmol) in DMF (15 ml), and the funnel was rinsed with precooled DMF (3 ml). The solution was allowed to warm to −15°, and was stirred at this temperature for 1 hr. The reaction mixture was kept at 4° overnight and then concentrated *in vacuo*, and the residue was dried. The residue was dissolved in H_2O (20 ml) and freed from insoluble material by filtration. The filtrate and washings (30 ml) were applied to a column of Dowex 1X8 resin (40 × 2.5 cm, acetate cycle) and eluted with H_2O. The Sakaguchi positive part of the eluate (from 60–150 ml) was collected, lyophilized, and dried to give 3.7 g (89%) of crude product, tlc R_f B 0.51, R_f D 0.46 (an additional spot, R_f B 0.6, R_f D 0.6).

A sample (250 mg, from an earlier preparation) was dissolved in 0.05 *M* ammonium acetate (15 ml) and applied to a column of carboxymethylcellulose (Bio Rad Cellex-CM, 2.5 × 25 cm). The column was washed with 0.05 *M* ammonium acetate (50 ml) and fractions (150 drops, *ca.* 6 ml) were collected. It was then eluted, at a flow rate of 30 ml/hr, with a linear gradient from 0.05 *M* ammonium acetate (500 ml) to 0.5 *M* ammonium acetate (500 ml). Fractions 17–23 were combined and lyophilized to give 50 mg, tlc R_f B 0.6, R_f D 0.6. Fractions 26–36 were combined and lyophilized to give 190 mg (65%) of the purified product. For analysis, a sample was relyophilized several times from H_2O: mp 135–140° (sintering at 120°); $[\alpha]^{24}D$ −60° (*c* 1, methanol); tlc R_f B 0.51, R_f D 0.46; amino acid analysis, Lys, 1.1, Ala, 0.9, Pro, 1.1, Ser, 1.7, Gly, 0.9, Arg, 1.1, Val, 0.9.

Anal. Calcd for $C_{46}H_{82}N_{12}O_{17}$ (1075.2): C, 51.4; H, 7.69; N, 15.6. Calcd for $C_{46}H_{82}N_{12}O_{17}\cdot 4H_2O$: C, 48.2; H, 7.9; N, 14.7. Found: C, 48.0; H, 7.5; N, 15.0. Loss of weight on drying at 110°: calcd, 6.3%; found, 5.6%.

N^{α},N^{ϵ}-Bis-*tert*-butyloxycarbonyl-L-lysyl-L-alanyl-L-prolyl-L-serylglycyl-L-arginyl-L-valyl-L-serine Hydrazide Acetate (XVIII).—The octapeptide ester XVII (103 mg) was dissolved in MeOH (1.5 ml) and 97% hydrazine (100 mg) was added to the solution. After 24 hr, the solution was evaporated to dryness with a stream of N_2 and the residue was lyophilized twice from H_2O to give 98 mg: mp 121–126° (with gradual shrinking); $[\alpha]^{24}D$ −83° (*c* 0.5, H_2O); tlc R_f B 0.47, R_f D 0.30; amino acid analysis, Lys, 1.0, Ala, 1.0, Pro, 1.1, Ser, 2.0, Gly, 1.0, Arg, 1.0, Val, 0.9.

Anal. Calcd for $C_{45}H_{82}N_{14}O_{16}$: C, 50.3; H, 7.7; N, 18.2. Calcd for $C_{45}H_{82}N_{14}O_{16}\cdot 4H_2O$: C, 47.1; H, 7.9; N, 17.1. Found: C, 47.0; H, 7.5; N, 17.6. Loss of weight on drying at 110°: calcd, 6.3%; found, 5.4%.

***N*-*tert*-Butyloxycarbonyl-L-prolyl-*O*-benzyl-L-serylglycine Benzyl Ester (XXI).**—*O*-Benzyl-L-serylglycine benzyl ester hydrochloride (XX)[20] (3.7 g, 9.7 mmol) was dissolved in EtOAc (60 ml) and neutralized with TEA (1.36 ml, 9.7 mmol) at 0°. *tert*-Butyloxycarbonyl-L-proline (2.08 g, 9.7 mmol) and DCC (2.20 g, 10.7 mmol) were added successively, stirring was continued for 3 hr at 0°, and the reaction mixture was allowed to stand overnight. After the addition of AcOH (0.5 ml), the mixture was filtered and the filtrate was washed with 2% citric acid solution, brine, 5% $NaHCO_3$, and brine, successively. The EtOAc solution was dried over $MgSO_4$ and evaporated *in vacuo* to give a sirupy residue (5.5 g). The crude product (4.5 g) was dis-

(20) T. Hayakawa, K. Harada, and S. W. Fox, *Bull. Chem. Soc. Jap.*, **39**, 391 (1966).

tributed through 100 transfers in the solvent system $CHCl_3$–toluene–MeOH–H_2O (5:5:8:2). The purified tripeptide (3.35 g) was isolated from a band with $k = 0.20$, yield 78%. The distribution curve was identical with the one calculated for $k = 0.20$; $[\alpha]^{23}D$ −27° (*c* 2, EtOAc); tlc R_f B 0.74, R_f E 0.59.

Anal. Calcd for $C_{29}H_{37}N_3O_7$ (539.6): C, 64.6; H, 6.9; N, 7.8. Found: C, 64.6; H, 7.1; N, 7.8.

L-Prolyl-*O*-benzyl-L-serylglycine Benzyl Ester Trifluoroacetate (XXII).—The *tert*-butyloxycarbonyl group was removed from compound XXI (3.0 g) as described for compound IV to give the trifluoroacetate XXII (2.8 g, 91%; tlc R_f B 0.48). A sample was reprecipitated from EtOH–ether. Its melting point was unchanged.

Anal. Calcd for $C_{26}H_{30}N_3O_7F_3$ (553.6): C, 56.4; H, 5.5; N, 7.6; F, 10.3. Found: C, 56.3; H, 5.6; N, 7.6; F, 10.2.

***N*-*tert*-Butyloxycarbonyl-L-alanyl-L-prolyl-*O*-benzyl-L-seryl-glycine Benzyl Ester (XXIII).**—*tert*-Butyloxycarbonyl-L-alanine *p*-nitrophenyl ester[10] (0.51 g, 1.65 mmol) was added to a suspension of XXII (1.0 g, 1.8 mmol) in $CHCl_3$ (25 ml) neutralized under ice cooling with TEA (0.25 ml, 1.64 mmol). The solution was stirred for 16 hr at room temperature and was kept slightly alkaline by the addition of TEA. After the evaporation of the solvent *in vacuo*, the residue was taken up in EtOAc, washed with 1 *M* NH_4OH, H_2O, 2% citric acid solution, and brine, and dried over $MgSO_4$. Evaporation gave a colorless, sirupy product which, on storage *in vacuo*, turned into an amorphous powder: 870 mg (87%); tlc R_f B 0.69, R_f E 0.49.

For further characterization, XXIII was converted into the corresponding hydrazide. A sample of XXIII (114 mg) and 97% hydrazine (100 mg) was dissolved in MeOH (2.0 ml) and allowed to react for 4 days at room temperature. After evaporation of the solvent, the residue was dried over P_2O_5 *in vacuo* and crystallized on trituration with ether. The residue was collected and washed with ether. Recrystallization from MeOH–ether gave 85 mg, mp 83–84°, $[\alpha]^{23}D$ −36° (*c* 1, MeOH).

Anal. Calcd for $C_{25}H_{38}N_6O_7$ (534.6): C, 56.2; H, 7.2; N, 15.7. Found: C, 55.8; H, 7.3; N, 15.6.

N^{α},N^{ϵ}-Bis-*tert*-butyloxycarbonyl-L-lysyl-L-alanyl-L-prolyl-L-serylglycine Pentachlorophenyl Ester (XXVII).—The protected tetrapeptide benzyl ester XXIII (2.0 g, 3.27 mmol) was dissolved in 95% TFA (3.0 ml) and allowed to react for 30 min. After evaporation of the TFA, addition of dry ether (50 ml) precipitated an oily product. The ether was removed by decantation and the residue was dried over NaOH pellets *in vacuo* to give an amorphous powder (2.04 g, 100%).

The trifluoroacetate XXIV (2.09 g, 3.34 mmol) was dissolved in EtOAc (35 ml) and neutralized under ice cooling with TEA (0.47 ml, 3.35 mmol). N^{α},N^{ϵ}-Bis-*tert*-butyloxycarbonyl-L-lysine *p*-nitrophenyl ester[11] (1.56 g, 3.33 mmol) was added, and the solution was stirred for 2 days at room temperature, while kept alkaline with the addition of a small amount of TEA. The product was taken up in EtOAc and treated as described for compound XXIII to yield an amorphous powder XXV (2.7 g, 96.4%): mp 59–66°; tlc R_f B 0.62, R_f E 0.54.

Compound XXV (2.1 g, 2.5 mmol) was dissolved in 95% EtOH (100 ml) and hydrogenated for 20 hr in the presence of a 10% Pd on charcoal catalyst (0.2 g). After removal of the catalyst, the solvent was evaporated *in vacuo*. The residue was dissolved in EtOAc (13 ml) and added to the complex[21] of pentachlorophenol (2.1 g, 7.9 mmol) and DCC (0.55 g, 2.7 mmol) in EtOAc (13 ml). The mixture was stirred at room temperature for 24 hr. After the addition of dry ether (30 ml), the residue was filtered and dissolved in dioxane (40 ml) and the DCU was removed by filtration. The filtrate was concentrated to dryness *in vacuo* and the residue was filtered with ether, yield 3.1 g. A sample (500 mg) was recrystallized from MeOH: 320 mg (82%); mp 167–173°; $[\alpha]^{23}D$ −35° (*c* 0.5, DMF); tlc R_f B 0.56, R_f E 0.65.

Anal. Calcd for $C_{35}H_{49}N_6O_{11}Cl_5 \cdot 3H_2O$ (961.1): C, 43.7; H, 5.8; N, 8.7; Cl, 18.4. Found: C, 43.7; H, 5.5; N, 8.8; Cl, 18.7. Loss of weight on drying at 110°: calcd, 5.6%; found, 5.1%.

Preparation of Compound XVII by the Active Ester Method.—L-Arginyl-L-valyl-L-serine methyl ester dihydrochloride (166 mg, 0.37 mmol), prepared as described earlier from protected tripeptide XIII, was suspended in DMF (3 ml) and neutralized with TEA (0.1 ml). The protected pentapeptide pentachlorophenyl ester (trihydrate) XXVII (363 mg, 0.37 mmol) was added to the solution and allowed to react for 40 hr, while the solution was kept alkaline with the addition of small amounts of TEA. The solvent was evaporated under a stream of N_2, and the residue was dissolved in $CHCl_3$ and extracted with H_2O. The aqueous layer was washed with $CHCl_3$ and EtOAc, and evaporated *in vacuo*. The residue was dissolved in H_2O, adsorbed in a column of Dowex 1X8 (acetate cycle, 40 × 2.5 cm), and eluted with distilled H_2O. The Sakaguchi positive fractions (60–140 ml) were pooled and lyophilized and the residue was dried *in vacuo*, yield *ca.* 250 mg. The residue was dissolved in a mixture of $CHCl_3$ and MeOH (1:1, 15 ml) and the filtered solution was applied to a column of silica gel (10 g, Baker 1.1 × 20 cm). The column was eluted first with the same solvent mixture (100 ml) and then with $CHCl_3$–MeOH (1:4, 100 ml). Compound XVII (50 mg) was recovered from the 1:1 mixture of the solvents, while the 1:4 mixture yielded some unchanged tripeptide ester XIV. The protected octapeptide ester was indistinguishable (tlc) from samples of XVII prepared by the azide method.

Registry No.—I, 34578-26-2; II, 34578-27-3; III, 34578-28-4; IV, 34578-29-5; V, 34578-30-8; VII, 34578-31-9; VIII, 34578-32-0; IX, 34578-44-4; X, 34578-33-1; XI, 34078-88-1; XIII, 34578-35-3; XVI, 34578-36-4; XVII, 34578-37-5; XVIII, 34578-38-6; XXI, 34578-39-7; XXII, 34578-40-0; XXIII hydrazide, 34578-41-1; XXV, 34578-42-2; XXVII, 34578-43-3; N^{α},N^{ϵ}-bis-*tert*-butyloxycarbonyl-L-lysyl-L-alanyl-L-prolyl-L-serylglycyl-L-arginine, 34578-45-5.

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