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Synthesis of *N*-Terminal Nonapeptide of Trypsinogen and Its Hydrolysis by Trypsin[1)]

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An *N*-terminal nonapeptide fragment of bovine trypsinogen, H-L-Val-L-Asp-L-Asp-L-Asp-L-Asp-L-Lys-L-Ile-L-Val-Gly-OH, was synthesized *via* two routes and its susceptibility with trypsin was investigated. Protected derivatives, *t*-butyloxycarbonyl protected nonapeptide *t*-butyl ester and benzyloxycarbonyl protected nonapeptide benzyl ester, were prepared by stepwise elongation. Removal of the protecting groups was carried out by treatment with trifluoroacetic acid and hydrogenation respectively. The naked peptide was purified with chromatography on ECTEOLA cellulose column using dilute acetic acid as a solvent. Upon lyophilization the desired nonapeptide was obtained as colorless powder, a portion of the product being digested with trypsin. Rate of tryptic hydrolysis of the peptide was found to be unexpectedly low in the absence of calcium ion, while considerable acceleration of the hydrolysis by calcium ion was noted.

Bovine trypsinogen is activated to trypsin upon cleavage with trypsin. An *N*-terminal hexapeptide of trypsinogen, H-Val-Asp-Asp-Asp-Asp-Lys-OH,[2)] is isolated on the cleavage and a tripeptide sequence of Ile-Val-Gly is found to be a newly generated *N*-terminal protion of trypsin.[3)] A similar tryptic cleavage of chymotrypsinogen furnishes the same Ile-Val-Gly sequence as a new *N*-terminal.[4)] Calcium ions seem to take part in this specific cleavage,[5)] but the mode of action of calcium has not yet been made clear. Savrda and Bricas[6)] prepared a related heptapeptide, H-Val-Asp-Asp-Lys-Ile-Val-Gly-OH, but the rate of tryptic digestion of this peptide was found to be rather low.[7)] As a cluster of four β-carboxylates in a tetra-aspartyl sequence adjacent to the cleaved bond was suggested to be the site of interaction of calcium ions with trypsinogen,[8)] a synthesis of *N*-terminal nonapeptide of trypsinogen, H-Val-$(Asp)_4$-Lys-Ile-Val-Gly-OH (I) was undertaken in order to clarify the role of the tetra-aspartyl carboxyl cluster in tryptic cleavage of the Lys-Ile bond.

Savrda and Bricas[9)] reported recently the synthesis of I and Abita *et al.*[10)] discussed the mode of tryptic digestion of I and related peptides. The rate of tryptic hydrolysis of I was found to be much lower than that of the previous heptapeptide,[7)] but it was raised three to four times by the addition of calcium ion. Radhakrishnan *et al.*[11)] reported that the role of calcium ion is in chelation to the tetra-aspartyl residues. These publications prompt us to disclose the present status of our study which includes synthesis of the nonapeptide I *via* two routes and tryptic digestion of synthesized I.

Reaction sequences employed for synthesis of I are shown in Figs. 1 and 2. In both routes, each protected amino acid was coupled with amino acid ester or peptide esters from *C*-terminus.

In the route of Fig. 1, *Z* group was used as α-amino protecting group of each amino acid other than *N*-terminal valine, and the Z group was selectively hydrogenolyzed in the presence of pal-

1) A part of this work was read at the 20th and 21st Meetings of the Chemical Society of Japan, Tokyo, April, 1967 and Osaka, April, 1968.

2) Abbreviations: Z-, benzyloxycarbonyl; BOC-, *t*-butyloxycarbonyl; Nps-, *o*-nitrophenylsulfenyl; Bz-, benzoyl; -OBu^t, *t*-butyl ester; -OBzl, benzyl ester; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; HPT, hexamethylphosphortriamide; *p*-TsOH, *p*-toluenesulfonic acid; DCHA, dicyclohexylamine. Amino acid symbols except Gly denote L-configuration.

3) E. S. Davide and H. Neurath, *J. Biol. Chem.*, **212**, 515 (1955); P. Desnuelle and C. Fabre, *Biochim. Biophys. Acta*, **18**, 29 (1955).

4) M. Rovely, M. Poilroux, A. Yoshida and P. Desnuelle, *ibid.*, **23**, 608 (1957).

5) M. R. MacDonald and M. Kunitz, *J. Gen. Physiol.*, **25**, 53 (1941).

6) J. Savrda and E. Bricas, *Bull. Soc. Chim. Fr.*, **1968**, 2423.

7) M. Delaage, P. Desnuelle and M. Lazdunski, *Biochem. Biophys. Res. Commun.*, **29**, 235 (1967).

8) T. M. Radhakrishnan, K. A. Walsh and H. Neurath, *J. Amer. Chem. Soc.*, **89**, 3059 (1967).

9) J. Savrda and E. Bricas, *Bull. Soc. Chim. Fr.*, **1969**, 883.

10) J. P. Abita, M. Delaage and M. Lazdunski, *Eur. J. Biochem.*, **8**, 314 (1969).

11) T. M. Radhakrishnan, K. A. Walsh and H. Neurath, *Biochemistry*, **8**, 4020 (1969).

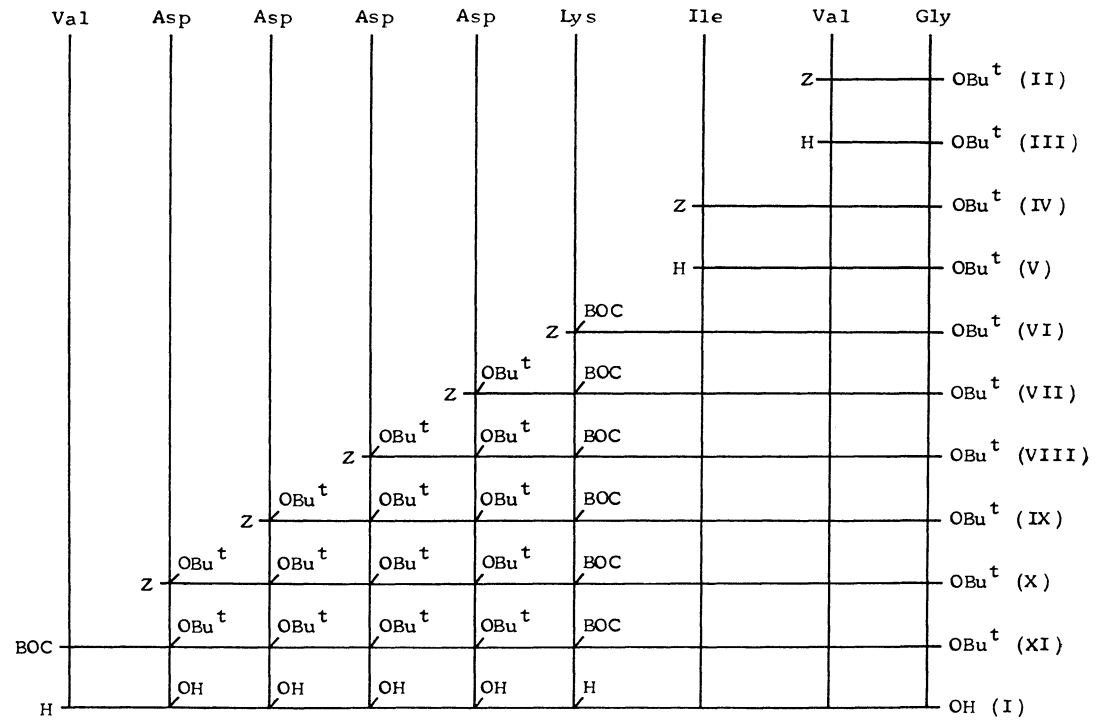

Fig. 1. Synthesis of the nonapeptide I using BOC and OBu^t protecting groups.

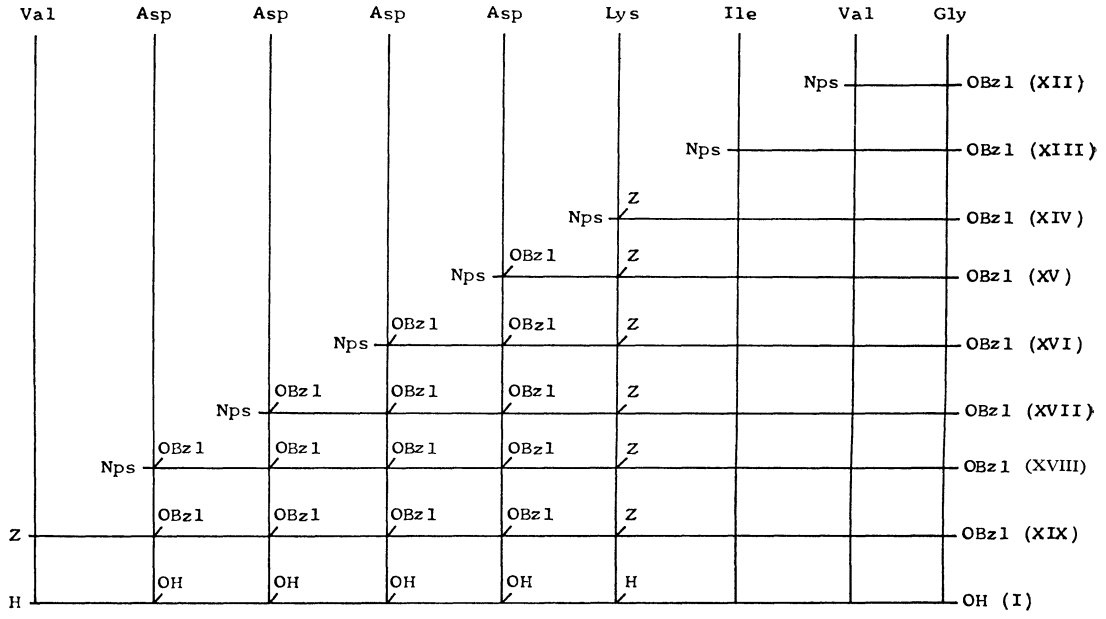

Fig. 2. Synthesis of I using Nps and OBzl groups.

ladium black. The protecting groups related to *t*-butyl alcohol were used for masking the side chain and the terminal functional groups. They are, BOC group for *N*-terminal amino group of valine and *ε*-amino group of lysine, and OBu^t group for *C*-terminal of glycine and *β*-carboxyl groups of four aspartic acid residues. In the coupling of each step, the mixed anhydride method using isobutylchloroformate as a coupling reagent generally gave good results. The use of 10—50% excess of each Z-amino acid brought satisfactory yield as in the cases of VII, VIII, IX and X. Z-

Val-Gly-OBu^t (II) was prepared with the esterification of Z-Val-Gly-OH[12] by the action of isobutylene in the presence of sulfuric acid as a catalyst. After hydrogenolysis of II, furnished dipeptide ester was coupled with the next amino acid, Z-Ile-OH. A similar procedure was repeated to attain the protected nonapeptide, BOC-Val-[Asp-(β-OBu^t)]$_4$-Lys(ε-BOC)-Ile-Val-Gly-OBu^t (XI), which was treated with trifluoroacetic acid to cleave all the protecting groups simultaneously. Although the BOC and OBu^t groups were reported previously to be easily cleavable,[13] all the acidolysis tested failed to give clear results. In addition to trifluoroacetic acid, hydrogen chloride in organic solvent, hydrogen bromide in acetic acid and liquid hydrogen fluoride were used as splitting reagents, but the product after acidolysis gave several ninhydrin-positive spots on their paper chromatograms and electrophoregrams. In order to purify the derived peptide from contaminations, chromatography on ECTEOLA cellulose column was found effective. Upon elution with dilute acetic acid, peptide I was obtained in a yield of about 30%. Anderson *et al.*[14] described the trifluoroacetic acid treatment to split five *t*-butyl esters from a protected macropeptide related to gastrin I and the chromatographic purification of the product, the over-all yield being lowered by the chromatographic procedure to about 10%.

Another route to synthesize the nonapeptide was planned in order to obtain I in better yield. In Fig. 2, Nps group[15] was used as α-amino protecting group except that of *N*-terminal valine. Z group and OBzl group were used to protect α-amino group of *N*-terminal valine, ε-amino group of lysine and all of the carboxyl groups, respectively, in order to hydrogenolyze all the protecting groups at once in the final stage of synthesis. Coupling of each protected amino acid with amino acid ester or peptide esters was accomplished *via* mixed anhydride method for syn-

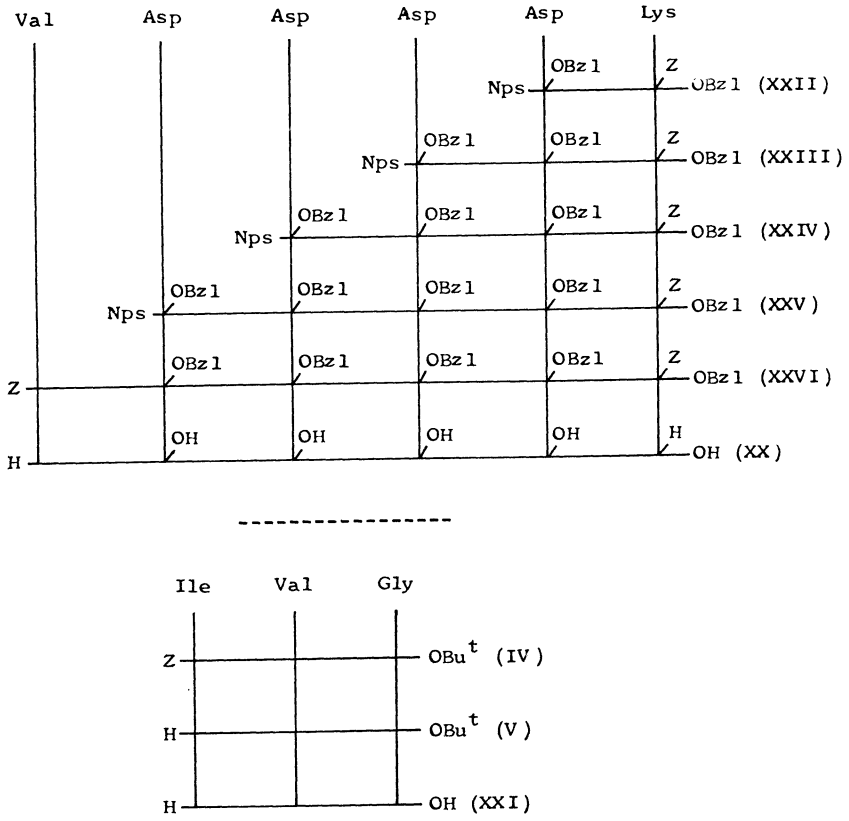

Fig. 3. Synthesis of the hexapeptide XX and tripeptide XXI.

12) E. Wünsch and A. Zwick, *Z. Physiol. Chem.*, **328**, 235 (1962).
13) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **44**, 1136, 1191 (1961).
14) J. C. Anderson, G. W. Kenner, J. K. Macleod and R. C. Sheppard, *Tetrahedron, Suppl.* **8**, part 1, 39 (1966).
15) L. Zervas, D. Borovas and E. Gazis, *J. Amer. Chem. Soc.*, **85**, 3660 (1963); L. Zervas and C. Hamalidis, *ibid.*, **87**, 99 (1965).

thesis of XIV—XIX, and *via* DCC procedure for XII and XIII. In the latter case, the desired acylpeptide ester was obtained easily in good yield by the procedure mixing up each Nps-amino acid DCHA salt with the corresponding amine component, such as H-Gly-OBzl·*p*-TsOH, and DCC. In the case of the mixed anhydride, the use of excess Nps- or Z-amino acid gave satisfactory yield similar to the previous instances. Removal of Nps group was brought about by treatment with N hydrogen chloride in dioxane for XII—XIV, in dioxane-DMF for XV, and in dioxane-dimethylsulfoxide for XVI and XVII.

Protected nonapeptide ester thus obtained, Z-Val-[Asp(β-OBzl)]$_4$-Lys(ε-Z)-Ile-Val-Gly-OBzl (XIX), was applied to a column of Sephadex LH-20 to remove supposed contamination of Nps derivatives. The purified XIX was, then, catalytically hydrogenated and the product was applied to ECTEOLA cellulose column chromatography to give pure peptide I tetrahydrate in a 40% yield. The protected nonapeptide ester was treated with hydrogen fluoride also, but no improvement could be observed compared to the result of hydrogenolysis.

Figure 3 shows syntheses of hexapeptide (XX) and tripeptide (XXI), as authentic samples on enzymic experiment. There was no trouble in synthesis of protected hexapeptide ester, Z-Val-[Asp-(β-OBzl)]$_4$-Lys(ε-Z)-OBzl (XXVI). After purification using Sephadex LH-20 chromatography, hydrogenolysis of XXVI gave peptide XX quantitatively in a pure state. Paper electrophoretic behavior of XX was the same as that of peptide I in citrate buffer at pH 6.7 (Fig. 4). As shown in Fig. 4, crude products from XI and XIX contain several ninhydrin positive components other than I, but these compounds were not identified.

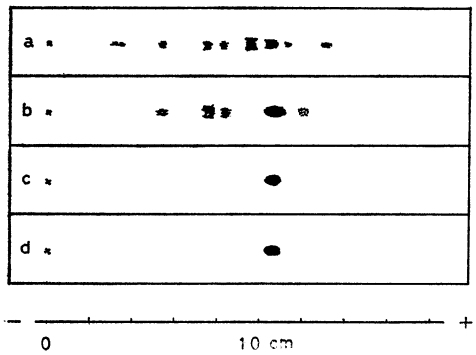

Fig. 4. Paper electrophoregrams of crude and pure peptides. Solvent, 0.01 M citrate buffer, pH 6.7; 600 V/30 cm; 1.5 hr. a, crude product by trifluoroacetic acid treatment on XI; b, crude product by hydrogenolysis of XIX; c, peptide I after ECTEOLA cellulose column chromatography; d, peptide XX.

H-Val-[Asp]$_4$-Lys-Ile-Val-Gly-OH (I)

↓ Trypsin

H-Val-[Asp]$_4$-Lys-OH (XX)

+ H-Ile-Val-Gly-OH (XXI)

Fig. 5. Scheme for tryptic cleavage of I.

Tryptic digestion of I (Fig. 5) was carried out in various conditions, and the progress of the reaction was followed by paper chromatography. In the absence of calcium ion, rate of tryptic hydrolysis of I was unexpectedly low at 30°C and the reaction was apparently inhibited at 47°C. On the contrary, when peptide I was digested with trypsin in the presence of 0.04 M calcium ion, the activating effect of calcium was always observed at 30°, 47° and 0°C, being especially remarkable at 47°C. In addition, susceptibility of I to trypsin was qualitatively compared with that of Bz-Arg-NH_2, one of the representative synthetic substrates of trypsin. For example, in a definite experimental condition described in Table 1, peptide I was digested by trypsin in 6 hr, while Bz-Arg-NH_2 was digested by one twentieth amount of trypsin in the same 6 hr interval. The results indicate that calcium ion accelerates tryptic digestion of I especially in high temperature as shown by Sipos and Merkel,[16] but the rate of digestion of I was found to be considerably low. This conclusion was compatible with the view given by Abita *et al.*[10] It should be noted that a similar promotion of hydrolysis of *N*-terminal undecapeptide of trypsinogen with calcium ion was also observed by Radhakrishnan *et al.*[11]

Experimental

Melting points were not corrected. Optical rotations were measured on a Yanagimoto Photometric Polarimeter OR-20 type. Paper chromatography and electrophoresis were carried out on Toyo Roshi No. 52 paper. Merck silica gel G was used for thin-layer chromatography. Developing solvents were *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2, v/v) for paper and chloroform-methanol (5 : 1, v/v) for thin-layer except noted otherwise.

Z-Val-Gly-OBu^t (II). To a solution of Z-Val-Gly-OH[13] (2.53 g, 8.4 mmol) and sulfuric acid (0.3 ml) in methylene chloride (100 ml), isobutylene (10.7 g) was saturated at 0°C. The mixture in a vessel with a tight stopper was kept to stand for 2 days at room temperature and evaporated *in vacuo*. The residue was diluted with ethyl acetate (100 ml) and the solution was washed successively with water, 4% sodium bicarbonate solution and saturated sodium chloride solution, and then dried over sodium sulfate. It was evapo-

16) T. Sipos and J. R. Merkel, *Biochem. Biophys. Res. Commun.*, **31**, 522 (1968).

rated *in vacuo* and the crystals were collected by filtration with the aid of ether and petroleum ether. Recrystallization from ethyl acetate-ether-petroleum ether gave 2.64 g (86%); mp 146°C; $[\alpha]_D^{30}$ −4.2° (*c* 1, DMF), (Found: C, 62.92; H, 7.91; N, 7.68%). Savrda *et al.*[6] prepared II by the coupling of Z-valine *p*-nitrophenyl ester with H-Gly-OBu^t; mp 143.5—144.5°C; $[\alpha]_D^{20}$ −2.5±0.5° (DMF).

H-Val-Gly-OBu^t (III). A solution of II (2.55 g, 7 mmol) in ethanol (80 m*l*) was hydrogenated in the presence of palladium black. After removal of the catalyst, the filtrate was evaporated *in vacuo*; yield of an oil, 1.60 g (99%); R_f 0.90 on paper.

Z-Ile-Val-Gly-OBu^t (IV). This compound was prepared by the coupling of III with a mixed anhydride derived from Z-Ile-OH and isobutylchloroformate as described in literature;[17] yield, 67%; mp 164—166°C, $[\alpha]_D^{20}$ −7.9° (*c* 1, DMF) (Found: C, 62.73; H, 8.29; N, 8.62%). Savrda *et al.* reported essentially the same procedure; mp 169—171°C, $[\alpha]_D^{20}$ −9±0.5° (DMF).[6]

H-Ile-Val-Gly-OBu^t (V). A suspension of IV (1.67 g, 3.5 mmol) in ethanol (30 m*l*) was hydrogenated as described for III. Evaporation of the solvent gave oily product (1.26 g, 105%); R_f 0.95 on paper.

Z-Lys(ε-BOC)-Ile-Val-Gly-OBu^t (VI). Coupling of Z-Lys(ε-BOC)-OH with V *via* mixed anhydride procedure gave VI; yield, 91%; mp 202—203°C; $[\alpha]_D^{30}$ −16.9° (*c* 1, DMF) (Found: C, 61.03; H, 8.59; N, 9.85%); reported values mp 213—215°C and $[\alpha]_D^{22}$ −14.5±0.5° (DMF).[6]

Z-Asp(β-OBu^t)-Lys-(ε-BOC)-Ile-Val-Gly-OBu^t (VII). A mixture of VI (3.25 g, 4.6 mmol) in DMF (15 m*l*) was cooled at 0°C and hydrogenated. After hydrogen was passed for 15 min, chilled 0.39 N hydrogen chloride in methanol (13 m*l*, 5.1 mmol) was added. After the reaction had been completed, the filtrate from the catalyst was evaporated. The condensed DMF solution was used as it was in the next coupling. The hydrogenated tetrapeptide was coupled with 1.2 equivalent of mixed anhydride derived from Z-Asp(β-OBu^t)-OH.[18] Yield of VII, 91%; mp 211—212°C (decomp.); $[\alpha]_D^{30}$ −21.2° (*c* 0.8, DMF) (Found as hemihydrate: C, 59.67; H, 8.27; N, 9.58%); reported values, mp 226—228°C and $[\alpha]_D^{20}$ −17.5±0.5° (DMF).[6]

Z-[Asp(β-OBu^t)]$_2$-Lys(ε-BOC)-Ile-Val-Gly-OBu^t (VIII). A similar coupling procedure using 1.25 equivalent of the mixed anhydride gave VIII (84%); mp 208—212°C, $[\alpha]_D^{30}$ −22.6° (*c* 1, DMF) (Found as hemihydrate: C, 59.02; H, 8.26; N, 9.14%); reported values, 225—226°C and $[\alpha]_D^{18}$ −22.5±0.5° (DMF).[6]

Z-[Asp(β-OBu^t)]$_3$-Lys(ε-BOC)-Ile-Val-Gly-OBu^t (IX). A similar coupling procedure using 1.5 equivalent of the mixed anhydride gave IX (92%); mp 213—214°C (decomp.); $[\alpha]_D^{30}$ −23.4° (*c* 1, DMF) (Found: C, 59.40; H, 8.30; N, 9.28%); reported values mp 222—224°C and $[\alpha]_D^{23}$ −25.7±0.7° (DMF).[9]

Z-[Asp(β-OBu^t)]$_4$-Lys(ε-BOC)-Ile-Val-Gly-OBu^t (X). A similar procedure as described above gave X (93%); mp 211—213°C (decomp.); $[\alpha]_D^{30}$ −25.5° (*c* 1, DMF) (Found as hemihydrate: C, 58.42; H, 8.10; N, 9.17%); reported values, mp 224—225°C and $[\alpha]_D^{22}$ −28.9°±0.5° (DMF).[9]

BOC-Val-[Asp(β-OBu^t)]$_4$-Lys(ε-BOC)-Ile-Val-Gly-OBu^t (XI). A similar procedure using 1.5 equivalent of BOC-Val-OH gave XI (91%); mp 219—221°C (decomp.); $[\alpha]_D^{30}$ −27.8° (*c* 1, DMF) (Found for monohydrate: C, 57.07; H, 8.37; N, 9.66%); reported values, mp 249°C and $[\alpha]_D^{20}$ −29.2±0.5° (DMF).[9]

Homogeneity of XI was ascertained by column chromatography using Sephadex LH-20. A solution of XI (5 mg) in methanol (0.6 m*l*) was applied to a column (1.7×37 cm) of Sephadex LH-20, and the column was eluted with methanol at room temperature with a flow rate of one drop per 6 sec. Two milliliter fractions were collected, and a small amount of eluate in each test tube was spotted on a thin-layer plate. Compound on the plate was detected by spraying 47% hydrobromic acid and then ninhydrin. Only one peak (tube number 20—23) was obtained. The result showed the homogeneity of XI. Several di-Z derivatives of cyclic decapeptide were found to elute at the same position under similar experimental conditions.[19]

Nps-Val-Gly-OBzl (XII). To a chilled solution of Nps-Val-OH·DCHA salt[15] (18.1 g, 40 mmol) and H-Gly-OBzl·*p*-TsOH (13.5 g, 40 mmol) in chloroform (80 m*l*), DCC[20] (8.24 g) was added. The mixture was stirred for 3 hr at 0°C and allowed to stand overnight at room temperature. After removal of precipitate by filtration, the filtrate was evaporated and the residual oil was dissolved in ethyl acetate (100 m*l*). The solution was washed with 4% sodium bicarbonate solution, 10% citric acid and water, and dried over sodium sulfate. The filtrate was evaporated and the oily residue was solidified upon addition of petroleum ether. Recrystallization from ether-petroleum ether gave 15.9 g (96%); mp 97—98°C, $[\alpha]_D^{22}$ +52.5° (*c* 2, DMF); R_f 0.90 on thin-layer.

Found: C, 57.40; H, 5.45; N, 10.09%. Calcd for $C_{20}H_{23}O_5N_3S$: C, 57.55; H, 5.55; N, 10.07%.

Nps-Ile-Val-Gly-OBzl (XIII). A solution of XII (10.4 g, 25 mmol) in dioxane (60 m*l*) was treated with 2.5 N hydrogen chloride in dioxane (40 m*l*) at room temperature. After 1 hr the mixture was evaporated and the crystals were washed with ether by decantation. The dipeptide ester hydrochloride (XIIa) (7.70 g, 103%; R_f 0.65 on thin-layer) obtained was used in the next procedure without further purification. Into a solution of Nps-Ile-OH·DCHA salt[15] (11.7 g, 25 mmol), triethylamine (3.5 m*l*) and XIIa in chloroform (140 m*l*), was added DCC (5.15 g) at 0°C. Usual coupling procedure as described for XII gave crude crystals of XIII. The crystals were collected and recrystallized from DMF-dioxane-ethyl acetate. Yield, 11.4 g (86%); mp 185—186°C; $[\alpha]_D^{22}$ +20.0° (*c* 2, DMF); R_f 0.88 on thin-layer.

Found: C, 58.92; H, 6.45; N, 10.44%. Calcd for $C_{26}H_{34}O_6N_4S$: C, 58.85; H, 6.46; N, 10.56%.

Nps-Lys(ε-Z)-Ile-Val-Gly-OBzl (XIV). Compound XIII (8.50 g, 16 mmol) was treated with 2.5 N hydrogen chloride in dioxane to furnish tripeptide hydrochloride (XIIIa) in a similar manner as described

17) J. R. Vaughan, Jr., and R. L. Osato, *J. Amer. Chem. Soc.*, **74**, 676 (1952).

18) P. M. Bryant, R. H. Moore, P. T. Pimlot and G. T. Young, *J. Chem. Soc.*, **1959**, 3868.

19) M. Waki and N. Izumiya, This Bulletin, **40**, 1687 (1967).

20) J. C. Sheehan and G. P. Hess, *J. Amer. Chem. Soc.*, **77**, 1067 (1955).

for XIII. Into a mixed anhydride of Nps-Lys(ε-Z)-OH[15] (7.51 g, 16.8 mmol) and isobutylchloroformate (2.18 ml) in tetrahydrofuran (100 ml), a solution of XIIIa (6.67 g, 16 mmol; R_f 0.71 on thin-layer) and triethylamine (2.24 ml) in DMF (120 ml) was added at −5°C. After being left overnight at room temperature, the solution was poured into water (1000 ml) and the precipitate deposited was collected by filtration, washed, and recrystallized from DMF-ethyl acetate. Yield, 11.4 g (90%); mp 223—225°C; $[\alpha]_D^{22}$ +23.0° (*c* 2, DMF); R_f 0.81 on thin-layer.

Found: C, 60.71; H, 6.62; N, 10.55%. Calcd for $C_{40}H_{52}O_9N_6S$: C, 60.59; H, 6.61; N, 10.60%.

Nps-Asp(β-OBzl)-Lys(ε-Z)-Ile-Val-Gly-OBzl (XV). Compound XIV (6.28 g, 7.9 mmol) was treated with 2.5 N hydrogen chloride in dioxane to give corresponding hydrochloride (XIVa). A solution of XIVa (4.99 g, 93%; R_f 0.61 on thin-layer) and triethylamine (1.02 ml) in DMF (55 ml) was added to a mixed anhydride of Nps-Asp(β-OBzl)-OH[15] (3.03 g, 8.04 mmol) in tetrahydrofuran (30 ml). The product was treated as described for the preparation of XIV. Recrystallization from DMF-ethyl acetate-ether gave 5.79 g (80%); 231—232°C; $[\alpha]_D^{22}$ −36.0° (*c* 1, HPT); R_f 0.88 on thin-layer.

Found: C, 61.38; H, 6.44; N, 9.77%. Calcd for $C_{51}H_{63}O_{12}N_7S$: C, 61.37; H, 6.36; N, 9.82%.

Nps-[Asp(β-OBzl)]$_2$-Lys(ε-Z)-Ile-Val-Gly-OBzl (XVI). A suspension of XV (4.24 g, 4.25 mmol) in DMF (120 ml) was treated with 2.5 N hydrogen chloride in dioxane (6.7 ml). The pale yellow crystals (3.74 g, 100%; R_f 0.74 on thin-layer) obtained were coupled with Nps-Asp(β-OBzl)-OH (1.93 g, 5.1 mmol) as described above. Recrystallization from dimethylsulfoxide-methanol-ether gave 4.02 g (79%); mp 222—223°C; $[\alpha]_D^{22}$ +15.6° (*c* 1, HPT); R_f 0.79 on thin-layer.

Found: C, 61.77; H, 6.49; N, 9.27%. Calcd for $C_{62}H_{74}O_{15}N_8S$: C, 61.88; H, 6.20; N, 9.31%.

Nps-[Asp(β-OBzl)]$_3$-Lys(ε-Z)-Ile-Val-Gly-OBzl-(XVII). A solution of XVI (3.39 g, 2.82 mmol) in dimethylsulfoxide (10 ml) was treated with 2.5 N hydrogen chloride in dioxane (7.0 ml). The hexapeptide ester hydrochloride (3.06 g, 100%; R_f 0.59 on thin-layer) obtained was condensed with a mixed anhydride of Nps-Asp(β-OBzl)-OH (1.37 g, 3.64 mmol) in the usual manner. Recrystallization from dimethylsulfoxide-methanol gave 3.19 g (81%); mp 214—216°C; $[\alpha]_D^{22}$ +21.6° (*c* 1, HPT); R_f 0.88 on thin-layer.

Found: C, 61.97; H, 6.35; N, 9.09%. Calcd for $C_{73}H_{85}O_{18}N_9S$: C, 62.24; H, 6.08; N, 8.95%.

Nps-[Asp(β-OBzl)]$_4$-Lys(ε-Z)-Ile-Val-Gly-OBzl (XVIII). Compound XVII (1.15 g, 0.82 mmol) was treated with 2.5 N hydrogen chloride in dioxane; yield of hydrochloride (XVIIa), 1.06 g, 100%; R_f 0.64 on thin-layer. The crude product obtained from XVIIa and Nps-Asp(β-OBzl)-OH (433 mg, 1.15 mmol) was recrystallized from dimethylsulfoxide-methanol. Yield, 1.19 g (91%); mp 221—224°C; $[\alpha]_D^{22}$ +22.4° (*c* 1, HPT); R_f, 0.74 on thin-layer.

Found: C, 62.20; H, 6.44; N, 8.48%. Calcd for $C_{84}H_{96}O_{21}N_{10}S$: C, 62.52; H, 6.00; N. 8.68%.

Z-Val-[Asp(β-OBzl)]$_4$-Lys(ε-Z)-Ile-Val-Gly-OBzl (XIX). Compound XVIII (1.10 g, 0.68 mmol) was treated with 2.5 N hydrogen chloride as described above. The pale yellow powder (1.02 g, 100%; R_f 0.65 on thin-layer) obtained was coupled with Z-Val-OH (256 mg, 1.02 mmol). Recrystallization from DMF-ethyl acetate gave light yellow product; 1.06 g (92%); mp 241—243°C; $[\alpha]_D^{22}$ +27.6° (*c* 1, HPT); R_f 0.64 on thin-layer.

Found: C, 62.84; H, 6.25; N, 8.41%. Calcd for $C_{91}H_{108}O_{22}N_{10}\cdot 2H_2O$: C, 63.17; H, 6.52; N, 8.09%.

A solution of XIX (80 mg) in DMF (1 ml) was applied to a column (0.9×40 cm) with Sephadex LH-20. Elution was carried out with the same solvent at room temperature. The flow rate was one drop per 7 sec, and 1.5 ml fractions were collected; only one peak (tube number 8—12) was observed.

H-Val-[Asp]$_4$-Lys-Ile-Val-Gly-OH (I). *a. I·4H_2O from XIX.* A solution of XIX (100 mg, 59 μmol) in a mixture of acetic acid (12 ml), methanol (6 ml), and water (2 ml) was hydrogenated in the presence of palladium black at room temperature. The progress of the reaction was followed by paper electrophoresis. After some 40 hr, the filtrate from the catalyst was evaporated repeatedly, water being added. The precipitate which formed upon the addition of ethanol was collected and dried. The yield was 67 mg. Forteen milligram of this was dissolved in 0.5 M acetic acid and it was applied to a column (0.9×10 cm) of ECTEOLA cellulose, which had been equilibrated with 0.05 M acetic acid. The column was eluted with 0.05 M (50 ml), 0.1 M (50 ml), 0.2 M (25 ml), 0.3 M (25 ml) and 0.5 M acetic acid. The combined eluate from No. 15—No. 18 tubes (2 ml each) was evaporated repeatedly at room temperature, water being added. After ethanol was added to the residue, the precipitate deposited was collected after standing in a refrigerator for several hours; yield, 4.8 mg (40% from XIX); mp 292—295°C (decomp.); $[\alpha]_D^{22}$ +36.4° (*c* 0.5, water); R_f 0.12 on paper; amino acid ratios in acid hydrolysate, $Asp_{3.86}Gly_{1.03}Val_{1.95}Ile_{1.00}Lys_{1.06}$.[21]

Found: C, 45.91; H, 6.92; N, 13.21%. Calcd for $C_{40}H_{66}O_{18}N_{10}\cdot 4H_2O$: C, 45.88; H, 7.12; N, 13.37%. Savrda *et al.*[9] described the preparation of I without elemental analysis by the action of trifluoroacetic acid on XI.

b. I from XI. A solution of XI (73.6 mg, 50 μmol) in a mixture of trifluoroacetic acid (1 ml) and anisole (0.5 ml) was kept for 2 hr at room temperature and the solution was evaporated *in vacuo*. This procedure was repeated, and the dried residue weighed 61.6 mg. Fifty milligram of this was dissolved in 0.5 M acetic acid (0.8 ml) and the solution was applied to a column of ECTEOLA cellulose (0.9×18 cm) as described for the preparation of I from XIX. The eluate from No. 15—No. 20 tubes was evaporated, and the precipitate was collected with the aid of ethanol. It was recrystallized from water-ethanol; 13 mg (30%); R_f 0.12 on paper.

Nps-Asp(β-OBzl)-Lys(ε-Z)-OBzl (XXII). The compound was prepared from Nps-Asp(β-OBzl)-OH·DCHA salt (1.67 g, 3 mmol) and H-Lys(ε-Z)-OBzl-*p*-TsOH (1.63 g, 3 mmol) using DCC as described for the preparation of XII. Yield, 1.82 g (84%); mp 127—129°C; $[\alpha]_D^{22}$ −17.2° (*c* 1, DMF); R_f 0.91 on thin-layer.

Found: C, 62.58; H, 5.51; N, 7.83%. Calcd for $C_{38}H_{40}O_9N_4S$: C, 62.62; H, 5.53; N, 7.69%.

21) The authors are indebted to Mr. Kosaku Noda of this Laboratory for the amino acid analysis.

Nps-[Asp(β-OBzl)]$_2$-Lys(ε-Z)-OBzl (XXIII). Compound XXII (1.33 g, 1.82 mmol) was treated with N hydrogen chloride in dioxane (7.3 ml) for 1 hr. The mixture was evaporated and treated with ether. Colorless crystals (1.07 g, 96%; *R_f 0.67 on thin-layer) obtained were coupled with Nps-Asp(β-OBzl)-OH (659 mg, 1.75 mmol) by the mixed anhydride procedure. Recrystallization from ethyl acetate-ether gave 1.40 g (84%); mp 137—138°C; $[\alpha]_D^{22}$ −1.4° (*c* 1, DMF); R_f 0.89 on thin-layer.

Found: C, 62.52; H, 5.42; N, 7.44%. Calcd for $C_{49}H_{51}O_{12}N_5S \cdot 1/2H_2O$: C, 62.41; H, 5.55; N, 7.43%.

Nps-[Asp(β-OBzl)]$_3$-Lys(ε-Z)-OBzl (XXIV). A solution of XXIII (1.78 g, 1.9 mmol) in N hydrogen chloride in dioxane (7.6 ml) was allowed to stand for 1 hr to give the tripeptide ester hydrochloride (1.29 g, 86%; R_f 0.79 on thin-layer), which was condensed with Nps-Asp(β-OBzl)-OH (746 mg, 1.97 mmol) as above. Yield, 1.61 g (89%); mp 123—125°C; $[\alpha]_D^{22}$ −25.0° (*c* 1, DMF); R_f 0.94 on thin-layer.

Found: C, 63.22; H, 5.50; N, 7.37%. Calcd for $C_{60}H_{62}O_{15}N_6S$: C, 63.25; H, 5.49; N, 7.38%.

Nps-[Asp(β-OBzl)]$_4$-Lys(ε-Z)-OBzl (XXV). Compound XXIV (365 mg, 0.32 mmol) was treated with hydrogen chloride as described above, and the corresponding hydrochloride (319 mg, 100%; R_f 0.67 on thin-layer) obtained was coupled with Nps-Asp(β-OBzl)-OH (196 mg, 0.52 mmol) as shown for XIV. Recrystallization from dioxane-ether gave 352 mg (82%); mp 143—145°C; $[\alpha]_D^{22}$ −32.0° (*c* 1, DMF); R_f 0.92 on thin-layer.

Found: C, 62.87; H, 5.39; N, 7.27%. Calcd for $C_{71}H_{73}O_{18}N_7S$: C, 63.42; H, 5.47; N, 7.29%.

Z-Val-[Asp(β-OBzl)]$_4$-Lys(ε-Z)-OBzl (XXVI). Nps group of compound XXV (310 mg, 0.23 mmol) was removed with hydrogen chloride in the usual manner, and the pentapeptide ester hydrochloride (XXVa, 250 mg, 0.205 mmol, R_f 0.97 on thin-layer) obtained was coupled with Z-Val-OH (75.4 mg, 0.3 mmol) by the mixed anhydride procedure. Recrystallization from dioxane-ethyl acetate gave pale yellow product; 258 mg (90%). A part (115 mg) of this product was applied to a column of Sephadex LH-20 (0.9×50 cm) using dioxane as a solvent. Fractions No. 7—No. 10 (2 ml each) were evaporated, and colorless crystals of XXVI were collected with the aid of ether. Recrystallization from dioxane-ether gave 94 mg (74% from XXVa); mp 151—158°C; $[\alpha]_D^{22}$ −37.0° (*c* 1, DMF); R_f 0.93 on thin-layer.

Found: C, 65.58; H, 5.96; N, 6.80%. Calcd for $C_{78}H_{88}O_{10}N_7$: C, 65.76; H, 6.01; N, 6.88%.

H-Val-[Asp]$_4$-Lys-OH (XX). A solution of XXVI (55 mg, 39 μmol) in a mixture of acetic acid (1.8 ml), methanol (0.9 ml), and water (0.3 ml) was hydrogenated in the presence of palladium black. After some 20 hr, the filtrate from the catalyst was evaporated repeatedly, water being added. The precipitate which formed upon the addition of ethanol was collected. Recrystallization from hot water-ethanol gave 24.7 mg (91%); mp 265—272°C(decomp.); $[\alpha]_D^{22}$ −38.0° (*c* 1, water); R_f 0.11 on paper; amino acid ratios in acid hydrolysate, $Asp_{3.91}Val_{1.00}Lys_{1.02}$[21]

Found: C, 43.16; H, 6.58; N, 12.99%. Calcd for $C_{27}H_{43}O_{15}N_7 \cdot 5/2\,H_2O$: C, 43.19; H, 6.45; N, 13.06%.

H-Ile-Val-Gly-OH (XXI). V from IV (0.5 mmol) was dissolved in trifluoroacetic acid (1.5 ml), and the solution was left to stand for 2 hr at room temperature and evaporated *in vacuo*. The residue dissolved in water (0.5 ml) was applied to a column of Dowex 50×8 (H^+ form, 0.9×7 cm). It was washed with water and eluted with 2 N ammonium hydroxide solution. The eluate was evaporated and the residue was collected with the aid of acetone. Recrystallization from water-acetone gave 122 mg (84%); mp 297—300°C (decomp.); $[\alpha]_D^{22}$ −16.0° (*c* 1, water); R_f 0.82 on paper.

Found: C, 53.77; H, 8.70; N, 14.53%. Calcd for $C_{13}H_{25}O_4N_3$: C, 54.33; H, 8.77; N, 14.62%.

Enzymatic Experiments. *a. Experimental Conditions.* Table 1 shows composition of reaction mixture used. Proceeding of enzyme reaction was followed

Table 1. Composition of mixture for the enzyme reaction

0.1 M Tris buffer at pH 8.0

Experiment number	Substrate (concentration, 0.01M)	Temperature (°C)	Calcium chloride concentration (M)	Enzyme concentration (mg N/ml)
1	peptide I	30	0.04	0.3
2	peptide I	30	0	0.3
3	peptide I	47	0.04	0.3
4	peptide I	47	0	
5	peptide I	0	0.04	0.3
6	peptide I	0	0	0.3
7	Bz-Arg-NH_2	30	0	0.3
8	Bz-Arg-NH_2	30	0	0.1
9	Bz-Arg-NH_2	30	0.04	0.1
10	Bz-Arg-NH_2	30	0	0.03
11	Bz-Arg-NH_2	30	0	0.015
12	Bz-Arg-NH_2	30	0	0.01
13	Bz-Arg-NH_2	30	0	0.0075
14	Bz-Arg-NH_2	30	0	0.006
15	Bz-Arg-NH_2	47	0.04	0.3
16	Bz-Arg-NH_2	47	0	0.3

by means of paper chromatography of each reaction mixture during the course of time. Developing solvent system used was *n*-butanol-acetic acid-pyridine-water (15 : 3 : 10 : 12, v/v).

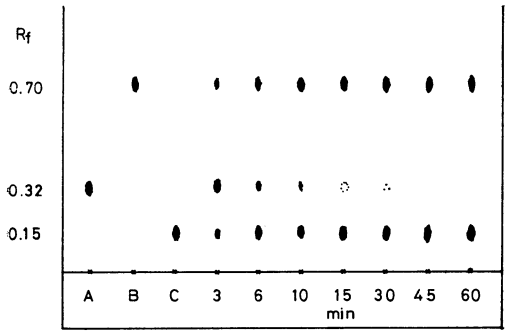

Fig. 6. Paper chromatograms of a reaction mixture of experiment No. 3 in Table 1. A, the nonapeptide I; B, the tripeptide XXI; C, the hexapeptide XX.

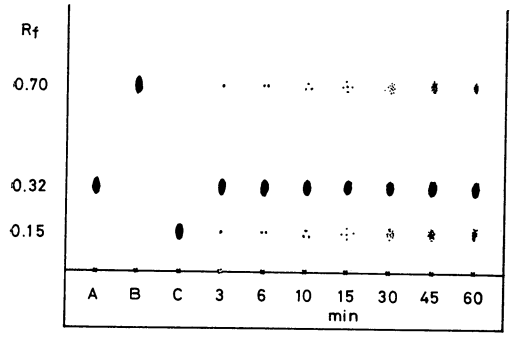

Fig. 7. Paper chromatograms of a reaction mixture of experiment No. 4. in Table 1.

b. Influence of Temperature Variation. Peptide I was digested with trypsin in the presence and absence of calcium ion at various temperatures. A comparison of the results of the experiments of No. 1—No. 6 indicates that the acceleration of hydrolysis with calcium was maximum at 47°C. Trypsin hydrolyzes peptide

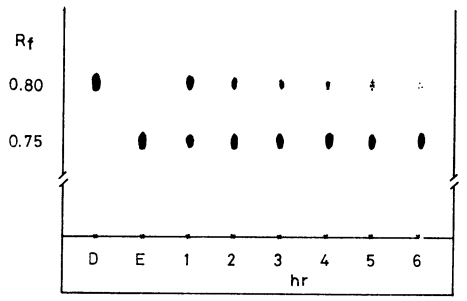

Fig. 8. Paper chromatograms of a reaction mixture of experiment No. 11 in Table 1. D, Bz-Arg-NH_2; E, Bz-Arg-OH.

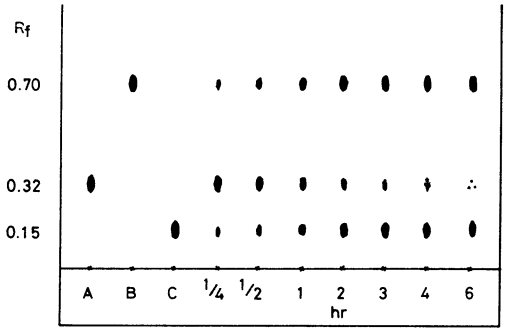

Fig. 9. Paper chromatograms of a reaction mixture of experiment No. 2 in Table 1.

I completely in 30 min with calcium (Fig. 6), but the hydrolysis was nearly inhibited without calcium (Fig. 7). At 30°C, complete digestion of I takes 2 hr and 6 hr in the presence and absence of calcium, respectively. At 0°C, peptide I disappeared after 14 hr with calcium, but it still remained after 19 hr without calcium.

c. Comparison of Hydrolysis Rates of I and Bz-Arg-NH_2. Experiments No. 7—No. 16 were carried out in order to compare the apparent rate of hydrolysis of I and Bz-Arg-NH_2, a typical synthetic substrate for trypsin. Experiment No. 11 (Fig. 8) showed that some one twentieth amount of trypsin was enough to hydrolyze Bz-Arg-NH_2 in the same time interval to hydrolyze I in experiment No. 2 (Fig. 9).