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Studies on Peptides. XXVII.[1,2] Synthesis of the Decapeptide, ACTH-(5-14), as an Example of Peptide-Fragment Condensation on the Polymer Support

HARUAKI YAJIMA, HIROKI KAWATANI and HIDEHIKO WATANABE

Faculty of Pharmaceutical Sciences, Kyoto University[3]

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As an example of peptide fragment condensation on the polymer support, synthesis of the tryptophan-containing decapeptide, ACTH-(5-14) was undertaken. First, suitable conditions to esterify Boc-Lys(Z)-Pro-Val-Gly-OH onto the copolymer of styrene and 2% divinylbenzene were examined. At room temperature, fairly good esterification of this tetrapeptide was achieved by the use of the bromomethylated copolymer and dicyclohexylamine. The peptide-resin was then treated with 1N HCl and triethylamine. To the resulting free tetrapeptide-resin, Z-Glu(OBzl)-His-Phe-Arg(NO_2)-Trp-Gly-OH was condensed by means of DCC and the growing peptide resin was treated with HF. The product, H-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-OH was obtained simply by column chromatography on CM-cellulose.

The copolymer, introduced by Merrifield[4] in the solid phase peptide synthesis, can be essentially classified as the protecting group of the carboxyl function of an amino acid in the form of insoluble benzyl ester. The chloromethylated copolymer of styrene and 1 or 2 % divinylbenzene is allowed to react with a triethylammonium salt of Boc-amino acid to form an acyl amino acid benzyl ester. Elongation of the peptide chain on the resin is performed by removing the α-amino protecting group with acid followed by condensation of succeeding amino acids one-at-a-time. The growing peptide-resin is insoluble in commonly used organic solvents. This property permits one to remove the unreacted carboxyl component, its by-product and excess reagents by simply washing the resin with appropriate solvents.

However, it should be realized that any by-product or an unreacted amino component attached on the resin can not be removed by such washing procedure and they will have another chance to react at each of the later step. When stepwise elongation of the peptide chain is progressed, accumulation of such impurity will result in the formation of a mixture of peptides of similar molecular size bearing similar properties. This gives serious problem in the purification of the desired product. Assessment of the purity of large molecular weight substances, such as insulin,[5] ribonuclease A,[6] apoferredoxin[7] or cytochrome c-like compound[8] by the usual bioassay technique is inaccurate, since the possibility can not be excluded that fragments of the protein or proteins with small differences in sequence may also possess the desired

1) Part XXVI: H. Yajima, H. Kawatani and H. Watanabe, *Chem. Pharm. Bull.* (Tokyo), **18**, 1279 (1970).
2) Amino acids, peptides and their derivatives are of the L-configuration. Abbreviations used are those recommended by IUPAC-IUB Commission on Biochemistry Nomenclature in July 1965 and July 1966; *Biochemistry*, **5**, 2485 (1966) and **6**, 362 (1967). Boc=*tert*-butoxycarbonyl, Z=benzyloxycarbonyl, OBzl=benzyl ester, ONp=*p*-nitrophenyl ester, For=formyl.
3) Location: *Sakyo-ku, Kyoto.*
4) R.B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963); *Biochemistry*, **3**, 1385 (1964); R.B. Merrifield and J.M. Stewart, *Nature*, **207**, 522 (1965); *Science*, **150**, 178 (1965); R.B. Merrifield, J.M. Stewart and N. Jernberg, *Anal. Chem.*, **38**, 1905 (1966).
5) A. Marglin and R.B. Merrifield, *J. Am. Chem. Soc.*, **88**, 5051 (1966); S. Hornle, *Z. Physiol. Chem.*, **348**, 1355 (1967).
6) B. Gutte and R.B. Merrifield, *J. Am. Chem. Soc.*, **91**, 501 (1969).
7) E. Bayer, G. Jung and H. Hagenmaier, *Tetrahedron*, **24**, 4853 (1968).
8) S. Sano and M. Kurihara, *Z. Physiol. Chem.*, **350**, 1183 (1969).

biological activity. It has been pointed out also that the result of amino acid analysis did not support the criteria of homogeneity of the final product.[9] Our experience in the solid phase synthesis of the eicosapeptide related to parathyroid hormone[1] indicated that there are certain limitations in the present solid phase synthesis with regard to its reproducibility.

If a pure acyl peptide with a certain chain length can be condensed onto the copolymer, instead of a single amino acid, to elongate the peptide chain, the above mentioned detriments in the present solid phase synthesis can be overcome in certain degree. Since, even if quantitative coupling of succeeding peptides can not be achieved, the property and the size of the resulting products may be quite different from those of the desired compound. Separation of the product will not be difficult. The unique property of the insoluble benzyl ester is maintained to allow the purification of the desired compound from the contamination of the acyl carboxyl component and coupling reagent. It should be emphasized that separation of these impurities causes major technical difficulty in the classical peptide synthesis.

In addition, this fragment condensation procedure is considered to be a rational method of choice for the synthesis of acid sensitive tryptophan-peptides, since by this procedure, a number of steps of acidolysis required for the deblocking of the amino protecting group can be reduced compared to the stepwise procedure. It is known, as we have also demonstrated, that the solid phase synthesis of tryptophan peptides can be achieved in certain degree by the use of mercaptoethanol.[1,10,11] But such modification has no promise in the synthesis of large molecules.

At present, only limited informations are available in such a fragment condensation reaction on the polymer support. An elegant example of this type of reaction was offered by Sakakibara, *et al.*[12] in the synthesis of a collagen model peptide. In which, Boc–Pro–Pro–OH was coupled with the H–Gly–resin. Subsequently, Boc–Pro–Pro–Gly–OH was repeatedly condensed up to the molecular weight of approximately 5,000. Condensation of acyldipeptides with H–Val–resin or with H–Ala–Ala–resin is an another example offered by Weygand, *et al.*[13] for the racemization test. For condensation of different subunits, Omenn, *et al.*[14] and Izumiya[15] offered preliminary informations about the reactivity on polymer support which depends on the molecular size or the nature of the terminal amino acid residues. Recent synthesis of valinomycin,[16] a cyclodecadepsipeptide, can be classified as an example of this type of reaction. However it is noted that most of the above examples were involved in the coupling reaction of acylpeptides with the amino acid–resin or peptide–resin prepared by the stepwise manner.

As our initial investigation of this approach, we have performed the fragment condensation of the glycine terminal hexapeptide unit, Glu–His–Phe–Arg–Trp–Gly, with an insoluble benzyl ester of the tetrapeptide unit, Lys–Pro–Val–Gly. It was confirmed that, after one-step purification, the tryptophan–containing decapeptide, H–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Val–Gly–OH, ACTH–(5–14) (I), could be obtained easily by this new approach.

First, the ester bond formation between the acyltetrapeptide, Boc–Lys(Z)–Pro–Val–Gly–OH and the chloromethylated copolymer was examined. Usually esterification is carried out by heating a mixture of Boc–amino acid with the chloromethylated resin in the presence of

9) J. Meienhofer and Y. Sano, *J. Am. Chem. Soc.*, **90**, 2996 (1968).
10) G.R. Marshall, *Advan. Exper. Med.*, **2**, 48 (1969).
11) J. Blake and C.H. Li, *J. Am. Chem. Soc.*, **90**, 5882 (1968).
12) S. Sakakibara, Y. Kishida, Y. Kikuchi, R. Sakai and K. Kakiuchi, *Bull. Chem. Soc. Japan*, **41**, 1273 (1968).
13) F. Weygand and U. Rahnarsson, *Z. Naturforsch.*, **21b**, 1141 (1966); F. Weygand, "Peptide 1968," ed., E. Bricas, North Holland Pub. Co., Amsterdam, 1968, p. 183.
14) G.S. Omenn and C.B. Anfinsen, *J. Am. Chem. Soc.*, **90**, 6571 (1968).
15) N. Izumiya, *Tanpakushitsu, Kakusan, Koso*, **14**, 641 (1969).
16) B.F. Gisin, R.B. Merrifield and D.C. Tosteson, *J. Am. Chem. Soc.*, **91**, 2691(1969).

triethylamine (2: 1 : 2 equi-moles) in appropriate solvents, such as ethyl acetate or ethanol for 50 hr.[4] Our effort was focused on the establishment of the insoluble benzyl ester bond of the acyltetrapeptide without heating. When equi-moles of the above mentioned three components in a mixture of ethanol and dimethylformamide (DMF) were shaked at room temperature for 72 hr, the yield of the peptide ester was at best less than 1%. Replacement of triethylamine with dicyclohexylamine (DCHA) or addition of sodium bromide according to Vesa, *et al.*[17] did not result in any noticeable improvement in the yield.

The use of bromomethylated copolymer was then studied. The resin was prepared by the condensation of bromomethyl methyl ether[18] to the copolymer in the presence of stannic bromide. The quantity of the halogen atom attached on the resin was purposely limited to approximately 2 to 3 mM per gram of the resin according to Merrifield.[4] Recently, Tilak[19] prepared the bromomethylated resin from the chloromethylated copolymer by treatment with triethylammonium acetate followed by hydrogen bromide. When the freshly prepared copolymer was used for this purpose, esterification of the acyltetrapeptide proceeded much more efficiently than the chloromethylated one. In our present experiments, bromine consumption proceeded efficiently for 72 hr at room temperature and reached to approximately 12% of the initial bromine content of the resin. Further improvement was obtained by replacement of triethylamine with DCHA. In order to perform mild and efficient esterification of valuable peptide fragments, it is desirable to carry out the reaction with molar ratios of the peptide, DCHA and the halogen atom on the resin, 1 : 1 : 5—8, within 72 hr period at room temperature. By such procedure, the yield of the peptide ester, approximately 44 to 52%, was obtained. Quantitative esterification of the resin is to be avoided since over crowded peptides may interfere the next coupling reaction. The content of the peptide in the resin prepared by the above procedure, approximately 0.2 to 0.3 mM/g, was nearly equivalent to that of the usual Boc–amino acid–resin prepared by heating in the present solid phase synthesis.

Next, esterification between the Boc–tetrapeptide and the hydroxymethyl resin was examined. The resin was prepared from the chloromethylated copolymer by treatment with potassium acetate followed by saponification with sodium hydroxide according to Bodanszky, *et al.*[20] In this case, the coupling reagent, dicyclohexylcarbodiimide (DCC), could not establish the ester bond between these two components. Presumably owing to the predominant acylurea formation, the coupling reaction of Z–Lys(For)–Pro–Val–Gly–OH with H–Lys(For)–Lys(For)–NH_2 by this reagent is known to be unsuccessful.[21] Carbonyldiimidazol, instead of DCC, should be used as the coupling reagent as suggested.[20-22] However this approach was abandoned.

After ester bond formation through the bromomethylated resin, the resin was treated with triethylammonium acetate to convert the unreacted bromine atom to the acetoxy residue in order to avoid possible side reaction in the next coupling reaction. The elementary analysis indicated that some halogen atom was still bound to the resin even after this treatment. The presence of such less reactive halogen atom was also pointed by Green, *et al.*[23] A part of which may be the halogen atom of quarternary ammonium salt of the resin formed in the esterification step.[24]

17) V. Vesa and N. Dirvianskyte, *Liet. TSR Mokslu. Akad. Darb., Ser.*, B (2) (1968); *C.A.*, **70**, 20321d (1969).
18) L. Henry, *Chem. Ber.*, **26**, 933 (1893).
19) M.A. Tilak, *Tetrahedron Letters*, **1968**, 6323.
20) M. Bodanszky and J.T. Sheehan, *Chem. Ind.*, **1966**, 1597.
21) K. Hofmann, N. Yanaihara, S. Lande and H. Yajima, *J. Am. Chem. Soc.*, **84**, 4470 (1962).
22) M.A. Tilak and C.S. Hollinden, *Tetrahedron Letter*, **1968**, 1297; G. Losse and K. Neubert, *Z. Chem.*, **8**, 387 (1968).
23) B. Green and L.R. Garson, *J. Chem. Soc.*, *(C)*, **1969**, 401.
24) H.C. Beyerman, C.A.M.B.-Boonekamp, J.W. Van Zoest and D. van den Berg, "Peptides 1967," ed. H.C. Beyerman, A. van de Linde and W.M. van, den Brink, North-Holland Pub. Co. Amsterdam, 1967, p. 117.

The peptide resin was then treated with 1 N HCl in dioxane for 30 min to remove the Boc group and the resulting hydrochloride was subsequently neutralized with triethylamine. The free peptide resin was allowed to react with Z–Glu(OBzl)-His–Phe–Arg (NO_2–)-Trp–Gly–OH by means of DCC for 72 hr. Because of consumption of the acylhexapeptide unit by acylurea formation, at least three moles or more acylpeptide had to be used for the coupling reaction. During this period, a part of the resin was removed from the reaction flask and submitted to amino acid analysis. When theoretical amino acid ratios corresponding to the expected decapeptide was obtained in the hydrolysate, the resin was washed thoroughly with DMF and then treated with anhydrous hydrogen fluoride[25] to cleave the ester bond from the resin and to remove all protecting groups from the growing peptide: The Z group from Glu and Lys and the benzyl ester group from Glu. Anisole and Trp were used to prevent the possible alkylation during this deblocking procedure. The product, after converting to its acetate, was subsequently submitted to purification on a column of CM-cellulose. When the column was developed with pyridinium acetate buffers, an essentially single peak of the decapeptide, H–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Val–Gly–OH, was detected as shown in Fig. 1. Formation of the tryptophan–destruction product was negligible.

Next, the filtrate of the resin in the above coupling reaction was examined. When the content was hydrogenated and the product was submitted to column chromatography on CM-cellulose, a considerable amount of by-product, presumably the hexapeptide urea was detected

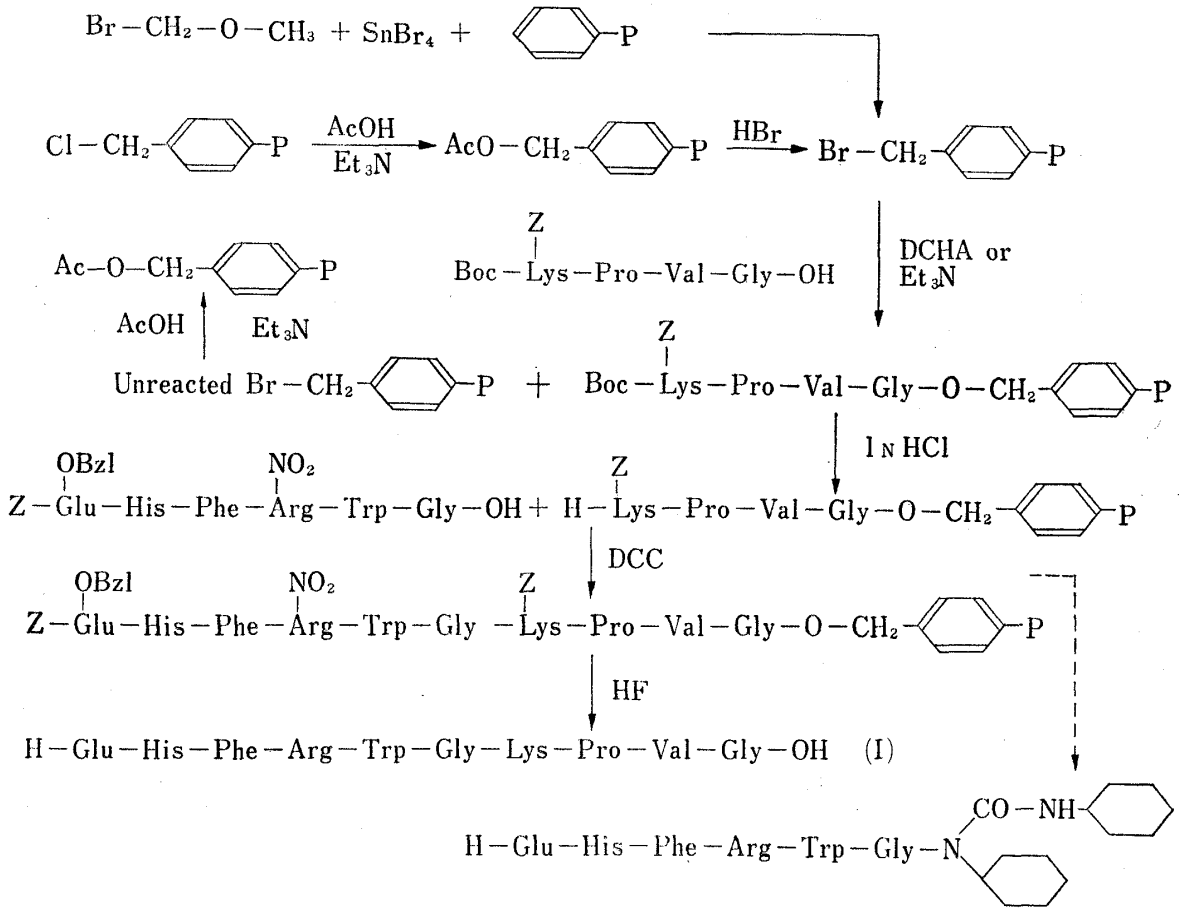

Chart 1. Synthesis of ACTH-(5-14) by the Fragment Condensation on the Polymer Support

25) S. Sakakibara and Y. Shimonishi, *Bull. Chem. Soc. Japan*, **38**, 1412 (1965); S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada and H. Sugihara, *ibid.*, **40**, 2164 (1967); S. Sakakibara, Y. Kishida, R. Nishizawa and Y. Shimonishi, *ibid.*, **41**, 438 (1968); J. Leonard and A.B. Robinson, *J. Am. Chem. Soc.*, **89**, 181 (1967).

as expected. The concentration of the pyridinium acetate buffer used for this elution was very close to that for the desired decapeptide (I) as shown in Fig. 2. It seems apparent that if the above coupling reaction would be performed by the classical condensation reaction, separation of the desired decapeptide from the acylurea might be a difficult task. By this comparison, it can be seen that in the reaction with the polymer support, the unreacted hexapeptide and the by-product were both removed simply by the previous washing procedure. The yield of the decapeptide in the present method, based on the tetrapeptide attached on the resin, was 40 to 45% in a range of three experiments.

We have offered here an example of the peptide fragment condensation reaction of two glycine–terminal peptides and demonstrated that the tryptophan–peptide could be synthesized without any sizable amount of destruction. For further development of this type of condensation reaction, which serves to reduce serious deficiency hitherto suffering both in the solid phase and classical procedures, examination of various means of condensation reaction as well as the suppressed racemization reaction of non-glycine terminal peptides on the polymer support seems to be required.

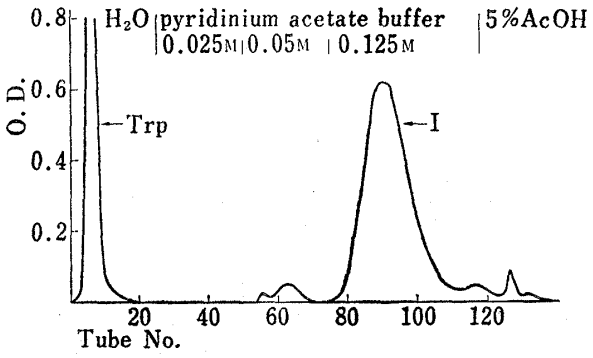

Fig. 1. Chromatographic Pattern of H–Glu–His–Phe–Arg-Trp–Gly–Lys–Pro–Val–Gly–OH prepared by the Fragment Condensation on the Polymer Support

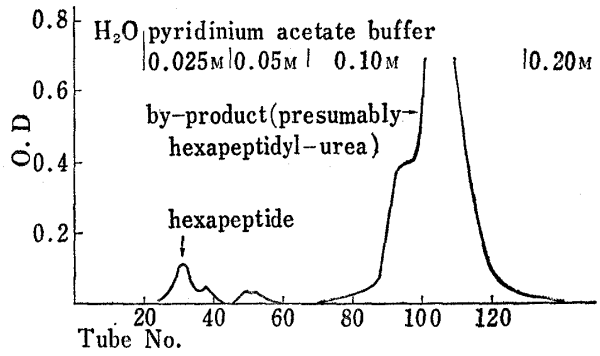

Fig. 2. Chromatographic Pattern of the By-product resulted from the Fragment Condensation on the Polymer Support

Experimental

For amino acid analysis, Hitachi Liquid Chromatography Model 003 was used. Thin-layer chromatography was performed on silica gel.

Boc-Lys(Z)-Pro-Val-Gly-OH——The title compound was prepared according to Fujino, *et al.*[26] mp 171—174°, $[\alpha]_D^{20}$ −69.2° (c=0.9, MeOH) (lit.[26] mp 174—175°, $[\alpha]_D^{22}$ −77.0° in MeOH). *Anal.* Calcd. for $C_{31}H_{47}O_9N_5$: C, 58.8; H, 7.5; N, 11.1. Found: C, 58.9; H, 7.4; N, 10.8.

Z-Glu(OBzl)-His-Phe-Arg(NO_2)-Trp-Gly-OH——Z–His–Phe–Arg(NO_2)–Trp–Gly–OH[27] (1.50 g) was treated with 27% HBr in AcOH for 1 hr. Dry ether was added and the resulting precipitate was washed with ether; yield 1.45 g. The hydrobromide thus obtained (0.73 g) was dissolved in DMF (10 ml) and triethylamine (0.28 ml) was added. To this solution, Z–Glu(OBzl)–ONp[28] (0.98 g) was added and the mixture was stirred for 24 hr. The solvent, after addition of a small amount of AcOH, was evaporated *in vacuo*. The residue was treated with AcOEt and the resulting solid powder was reprecipitated from DMF with AcOEt; yield 0.82 g (72%), mp 215—220°, $[\alpha]_D^{25}$ −21.8° (c=1.0, DMF). *Anal.* Calcd. for $C_{54}H_{61}O_{13}N_{13}\cdot 2H_2O$: C, 57.1; H, 5.8; N, 16.0. Found: C, 57.2; H, 5.7; N, 15.5.

Bromomethyl Methyl Ether——The title compound was synthesized according to the procedure of corresponding chloromethyl methyl ether;[29] yield 61%, bp 87—92° (lit.[18] bp 87—92°).

26) M. Fujino, O. Nishimura and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **17**, 2135 (1969).
27) K. Hofmann and S. Lande, *J. Am. Chem. Soc.*, **83**, 2286 (1961).
28) M. Goodman and K.C. Stuben, *J. Am. Chem. Soc.*, **81**, 3980 (1959); G. Losse, H. Jeschkeit, and W. Langenbeck, *Ber.*, **96**, 204 (1963).
29) C.S. Marvel and P.K. Porter, *Org. Syn.*, **1**, 377 (1948).

Bromomethylated Copolymer——The copolymer of styrene with 2% divinylbenzene (4.31 g, Bio-Beads S-X2, 200—400 mesh, control number 4818) was suspended in bromomethyl methyl ether (20 ml) at 0° for 1 hr. Stannic bromide (1.23 g) in bromomethyl methyl ether (10 ml) was added to the above suspension with gentle stirring. After 30 min, the resin was collected by filtration and washed with 200 ml each of dioxane–H_2O (3:1), dioxane–3N HBr (3:1), H_2O, dioxane and finally MeOH. The bromine content was determined by the Volhard method. The resin of the bromine content 1.8 to 3.2 mM/g was obtained. Alternatively, the chloromethylated resin (Cl, 1.6 mM/g, 2% divinylbenzene, 100—200 mesh) was converted to the corresponding bromomethylated resin according to Tilak.[19]

Esterification of Boc-Lys(Z)-Pro-Val-Gly-OH onto the Chloromethylated Copolymer——To a suspension of the chloromethylated copolymer (Cl, 1.6 mM/g, 100—200 mesh) in EtOH–DMF (1:1, 4 ml), Boc–Lys(Z)–Pro–Val–Gly–OH and triethylamine (Et_3N) or DCHA were added and the mixture was shaked at room temperature for 48 hr or 72 hr. The resin was then washed three times with the following solvents (5 ml each); EtOH–DMF (1:1), DMF, EtOH, H_2O and EtOH. A part of the dried resin was submitted to acid hydrolysis. The yield of the esterified peptide was calculated from the recovery of Lys.

Resin mg (Cl, 0.2 mM)	Base ml (0.2 mM)	Boc-4-OH mg (0.2 mM)	Condition 25—28°	Lys recovery mM/g	Yield %
125	Et_3N 0.027	126	48 hr	0.0057	0.36
125	Et_3N 0.027	126	72 hr	0.0098	0.61
125	DCHA 0.036	126	48 hr	0.0118	0.74
125	Et_3N 0.027 NaBr 80 mg	126	72 hr	0.0096	0.60

Esterification of Boc-Lys(Z)-Pro-Val-Gly-OH onto the Bromomethylated Copolymer——The entire operation was performed as described above using the bromomethylated copolymer (Br, 1.8 mM/g).

Resin mg (Br content)	Base ml	Boc-4-OH mg	Condition 25—28°	Lys recovery mM/g	Yield %
111 (0.2 mM)	Et_3N 0.027 (0.2 mM)	126 (0.2 mM)	48 hr	0.221	12.3
111 (0.2 mM)	Et_3N 0.027 (0.2 mM)	126 (0.2 mM)	72 hr	0.241	13.4
111 (0.2 mM)	DCHA 0.036 (0.2 mM)	126 (0.2 mM)	48 hr	0.257	14.3
111 (0.2 mM)	DCHA 0.036 (0.2 mM)	126 (0.2 mM)	72 hr	0.317	17.6
555 (1.0 mM)	DCHA 0.036 (0.2 mM)	126 (0.2 mM)	72 hr	0.185	51.3
1776 (3.2 mM)	DCHA 0.072 (0.4 mM)	253 (0.4 mM)	72 hr	0.118	52.4

Attempt to Esterify Boc-Lys(Z)-Pro-Val-Gly-OH onto the Hydroxymethyl Copolymer——According to Bodanszky, *et al.*,[20] the chloromethylated copolymer (5 g, Cl, 1.6 mM/g) was treated with potassium acetate (2.35 g, 24 mM) in benzylalcohol (20 ml) at 80° for 48 hr. The resulting acetoxymethyl resin (4.5 g, remaining Cl 0.17 mM/g) was treated with NaOH (0.86 g, 23 mM) in EtOH (20 ml) at 25° for 24 hr and then washed thoroughly with EtOH, H_2O and EtOH. The resin (0.57 g, Cl, *ca.* 0.17 mM/g) was suspended in methylene chloride (5 ml). To which, Boc–Lys(Z)–Pro–Val–Gly–OH (0.65 g, 1.02 mM) and DCC (0.21 g, 1.02 mM) were added and the mixture was shaked at room temperature for 24 hr. Acid hydrolysis of the washed and dried resin (37 mg) gave only negligible amounts of the constituent amino acids.

Treatment of the Esterified Resin with CH_3COOK or Triethylammonium Acetate——A sample of the esterified resin (0.5 g, peptide 0.185 mM/g, Br 1.1 mM/g) was treated with 1N triethylammonium acetate solution of DMF–EtOH (9:1, 2 ml) at room temperature for 72 hr. The bromine content of this sample was 0.7 mM/g. When esterified resin (77 mg, peptide 0.28 mM/g, Br 1.0 mM/g) was treated with CH_3COOK (50 mg) in DMF–MeOH (9:1, 2 ml) at room temperature for 72 hr, the remaining bromine was 0.3 mM/g.

H-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-OH (I)——Boc–Lys(Z)–Pro–Val–Gly–resin (550 mg, peptide 0.123 mM/g) treated with triethylammonium acetate as stated above, was swelled in dioxane (5 ml) and then treated with 1N HCl–dioxane (3 ml) for 45 min. The resin, after washing with dioxane (5 ml × 3) and DMF (5 ml × 3), was suspended in 1N triethylamine solution in DMF (5 ml) for 30 min and then collected by filtration. To the suspension of the resin in DMF (5 ml), Z–Glu(OBzl)–His–Phe–Arg(NO_2)–Trp–Gly–OH (270 mg, 4 equi-moles) and DCC (101 mg, 4 equi-moles) were added and the reaction flask was shaked at

30) E.C. Jorgensen, G.C. Windridge, W. Patton and T.C. Lee, *J. Med. Chem.*, **12**, 733 (1969).

room temperature for 72 hr. A part of the resin (35 mg) was branched off and submitted to analysis. Amino acid ratios of Arg and Lys were 0.96 and 1.01. The resin was washed thoroughly with DMF, AcOH, H_2O and MeOH (5 ml each) and dried. The dried resin (500 mg) was then treated with anhydrous hydrogen fluoride (approximately 10 ml) in the presence of anisole (0.3 ml) and Trp (15 mg) at 0° for 1 hr. Hydrogen fluoride was evaporated *in vacuo* and the residue, after drying over KOH pellets *in vacuo*, was dissolved in H_2O (20 ml). The solution was filtered with an aid of filter cell and the filtrate was then treated with Amberlite CG-4B (acetate cycle, approximately 5 g). The resin was removed by filtration and the filtrate was applied to a column of CM-cellulose (1.7×10 cm), which was developed with the following pyridinium acetate buffers (pH 5.0); 0.025M (300 ml), 0.05M (300 ml) and 0.125M (500 ml). The individual fractions (12 ml each) were collected and the absorbancy at 280 mμ was determined in each fraction. A single peak present in the 0.125M eluates (tube 80 to 113) was detected. Lyophilization of this fraction gave fluffy powder; yield 37 mg (40%, based on the tetrapeptide), $[\alpha]_D^{20}$ −55.2° (c=0.4, 5% AcOH). *Rf* 0.16 (*n*-BuOH:AcOH: pyridine:H_2O, 4:1:1:2), *Rf* 0.24 (*n*-BuOH–AcOH–H_2O, 4:1:5). Amino acid ratios in an acid hydrolysate $Glu_{0.97}His_{1.08}Phe_{0.98}Arg_{1.18}Gly_{2.00}Lys_{1.01}Pro_{1.02}Val_{0.94}$ (average recovery 91%). Amino acid ratios in a aminopeptidase (AP-M, Rohm & Haas Darmastadt) digestion $Glu_{1.07}His_{1.10}Phe_{0.98}Arg_{1.00}Trp_{1.29}Gly_{1.56}Lys_{\mp 0}$ $Pro_0Val_{0.66}$ (low recovery of Lys, Pro, Val and Gly is presumably due to the lack of the prolidase-like activity of this enzyme).[30] *Anal.* Calcd. for $C_{57}H_{81}O_{13}N_{17}\cdot 3CH_3COOH\cdot 7H_2O$: C, 49.8; H, 7.1; N, 15.7. Found: C, 49.1; H, 6.6; N, 15.4.

Examination of the Filtrate of the Coupling Reaction——After the coupling reaction of the hexapeptide onto the tetrapeptide resin, the resin was collected by filtration and washed with DMF as stated above. This filtrate was evaporated and the residue was treated with ether. The resulting powder (180 mg) was hydrogenated over a Pd catalyst in 70% AcOH (40 ml). The product was applied to a column of CM-cellulose (1.5×17 cm), which was eluted as described above. The elution pattern is shown in Fig. 2. The amino acid ratios of the acid hydrolysate of the product obtained in the 0.1M eluates were $Glu_{1.10}Gly_{1.00}$ $Phe_{0.91}$. Pro and Val were absent in the neutral column.

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