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Studies on Peptides. XXXII.[1,2] The Use of N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline as a Coupling Reagent of Peptide-Fragments on Polymer Support

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As an example of peptide-fragment condensation on the polymer support, coupling reaction between Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH (positions 19 to 24 of ACTH) and H-Asp (OBzl)-Gly-resin and H-Ala-Gly-resin was performed. Among various coupling reagents so far examined, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline gave relatively good results.

In the proceeding paper,[4] suitable conditions to esterify acylpeptides onto the copolymer of styrene and 2% divinylbenzene were examined and we have found that at room temperature fairly good esterification of a model peptide was achieved by the use of the bromomethylated copolymer and dicyclohexylamine (DCHA). In this paper, we wish to present the data that N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), introduced by Belleau and Malek[5] in 1968, seems to be a suitable reagent for condensation of acylpeptides with peptides anchored copolymer in order to elongate the peptide chain.

Only limited informations are now available in such a fragment condensation reaction on the polymer support. Dicyclohexylcarbodiimide (DCC) alone[4,6–8] or DCC puls N-hydroxysuccinimide (NHSI)[7,9,10] is the reagent commonly employed for this purpose. In addition, the NHSI ester of peptides was used by Weygand[9] and N-ethyl-5-phenylisoxazolium-3'-sulfonate (NEPIS) was examined by Omenn, *et al.*[7] On the basis of the amino component attached on the polymer, at least, three or four equimoles of the carboxyl component and the coupling reagent are employed in order to pursue the quantitative coupling reaction. However, despite of the use of such large excess of reagents, the reactivity of these reagents, as pointed out by Omenn, *et al.*,[7] seems to depend on the molecular size and the nature of the terminal amino acid residues involved in the coupling reaction.

Omenn, *et al.* offered example that the coupling the octa or tetrapeptide both containing COOH-terminal Val to the nonapeptide copolymer with the NH_2-terminal Asp gave only 20 to 30% coupling with either DCC or NEPIS. An additional example indicated also that the NH_2-terminal β-benzyl-Asp residue offered special difficulty.[7] Therefore, further funda-

1) Part XXXI: H. Yajima, N. Shirai and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **19**, 1900 (1971).
2) Amino acids, peptides and their derivatives mentioned in this communication are of the L-configuration. Abbreviations used are those recommended by IUPAC-IUBC Commission on Biochemistry Nomenclature in July 1965 and July 1966. *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967). Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Boc=*tert*-buto-xycarbonyl, ONp=*p*-nitrophenyl ester.
3) Location: *Sakyo-ku, Kyoto.*
4) H. Yajima, H. Kawatani and H. Watanabe, *Chem. Pharm. Bull.* (Tokyo), **18**, 1333 (1970).
5) B. Belleau and G. Malek, *J. Am. Chem. Soc.*, **90**, 1651 (1968).
6) S. Sakakibara, Y. Kishida, Y. Kikuchi, R. Sakai and K. Kakiuchi, *Bull. Chem. Soc. Japan.*, **41**, 1273 (1968).
7) G.S. Omenn and C.B. Anfinsen, *J. Am. Chem. Soc.*, **90**, 6571 (1968).
8) B.F. Gisin, R.B. Merrifield and D.C. Tosteson, *J. Am. Chem. Soc.*, **91**, 2691 (1969).
9) F. Weygand and U. Ragnarsson, *Z. Naturforsch.*, **21b**, 1141 (1966).
10) K. Noda, S. Terada, N. Mitsuyasu, N. Yoshida, T. Kato, M. Waki, S. Makisumi and N. Izumiya, "Proc. of the 7th Symp. on Peptide Chem.," Pub. by Protein Research Foundation in the Institute for Protein Research, Osaka University, 1969, p. 40.

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mental investigations seem to be required in the approach of fragment condensation reaction on the polymer support, which aims to obtain more pure peptides than that produced by the stepwise addition procedure.[11] The former approach reduces the number of coupling reactions required to construct the desired peptide chain and may permit us to isolate the desired peptide from contaminating peptides with incomplete sequences of different molecular sizes which will be accumulated on the copolymer.

In our present investigation, we pursued necessary conditions required for the quantitative coupling between the proline terminal pentapeptide unit, Val-Lys-Val-Tyr-Pro (corresponding to positions 19 to 24 of ACTH) and an insoluble benzyl ester of Asp-Gly (positions 25 to 26 of bovine ACTH)[12,13] or Ala-Gly (positions 25 to 26 of sheep ACTH).[14] Because of the risk of racemization which is always present when the carboxyl group of a protected peptide is activated, condensation of a fragment with COOH-terminal glycine or proline with appropriate amino components is a method of choice currently employed for the synthesis of racemization-free peptides.[15] Since an example of the fragment condensation of the glycine-terminal peptide on the polymer support was offered in our previous paper,[4] we are interested in examining a fragment with COOH-terminal proline at this time. With considerations of a comment mentioned by Omenn, *et al.*,[7] condensation reaction of a peptide fragment of ACTH which contains the aspartyl residue mentioned above can be considered as a suitable example of choice at our initial investigations.

First, three Z(OMe)-peptides, Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH (I), Z(OMe)-Asp (OBzl)-Gly OH (II) and Z(OMe)-Ala-Gly-OH (III), were synthesized. The Z(OMe) group, the reagent of which was prepared by our recently devised procedure,[16] can be removed by mild acidolysis such as by treatment with trifluoroacetic acid (TFA) like the Boc group commonly used in the solid phase synthesis.[11] Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH (I) was prepared by the coupling reaction of Z(OMe)-Val-Lys(Z)-azide with H-Val-Tyr-Pro-OH, which was prepared according to Ramachandran and Li.[17] The corresponding Boc-pentapeptide, Boc-Val-Lys(Z)-Val-Tyr-Pro-OH was also synthesized as a reference. Z(OMe)-Asp(OBzl)-Gly-OH (II) was prepared by condensation of Z(OMe)-Asp(OBzl)-OH[18] with H-Gly-OH by the mixed anhydride procedure[19] and Z(OMe)-Ala-Gly-OH (III) was obtained after the reaction of Z(OMe)-Ala-ONp with the triethylammonium salt of H-Gly-OH. These two dipeptides, II and III, were converted to the corresponding dicyclohexylamine (DCHA) salts respectively.

Previous esterification procedure[4] was slightly modified. As shown in Fig. 1, a suspension of the bromomethylated copolymer of styrene and 2% divinylbenzene (7 equimoles) and the DCHA salt of Z(OMe)-Asp(OBzl)-Gly-OH in DMF was shaken at 30° for 48 hr and then the resin was collected by filtration. This modification may eliminate the possibility of the

11) R.B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963); *idem*, *Biochemistry*, **3**, 1385 (1964); *idem*, *Advan. in Enzymology*, **32**, 221 (1969); A. Marglin and R.B. Merrifield, *Ann. Rev. Biochem.*, **39**, 841 (1969).
12) R.G. Shepherd, S.D. Willson, K.S. Howard, P.H. Bell, D.S. Davies, S.B. Davis, E.A. Eigner and N.E. Shakespeare, *J. Am. Chem. Soc.*, **78**, 5067 (1956).
13) C.H. Li, J.S. Dixon and D. Chung, *J. Am. Chem. Soc.*, **80**, 2587 (1958); *idem*, *Biochim. Biophys. Acta*, **46**, 324 (1961).
14) C.H. Li, I.I. Geschwind, R.D. Cole, I.D. Raacke, J.I. Harris and J.S. Dixon, *Nature*, **176**, 687 (1955); B.T. Pickering, R.N. Andersen, P. Lohmar, Y. Birk and C.H. Li, *Biochim. Biophys. Acta*, **74**, 763 (1963).
15) See review articles: E. Schröder and K. Lübke, "The Peptide," Academic Press, 1965, p 319; M. Bodanszky and M.A. Ondetti, "Peptide Synthesis," Pub. by J. Wiley Interscience Publisher, 1966, p 137.
16) H. Yajima and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **17**, 1962 (1969); H. Yajima, H. Kawatani and Y. Kiso, *ibid.*, **18**, 850 (1970).
17) J. Ramachandran and C.H. Li, *J. Org. Chem.*, **28**, 173 (1963).
18) H. Yajima and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **19**, 420 (1971); F. Weygand and E. Nintz, *Z. Naturforsch.*, **20b**, 429 (1965).
19) Th. Wieland and H. Bernhard, *Ann. Chem.*, **572**, 190 (1951); R.A. Boissonnas, *Helv. Chim. Acta*, **34** 874 (1951); J.R. Vaughan, Jr., *J. Am. Chem. Soc.*, **73**, 3547 (1951).

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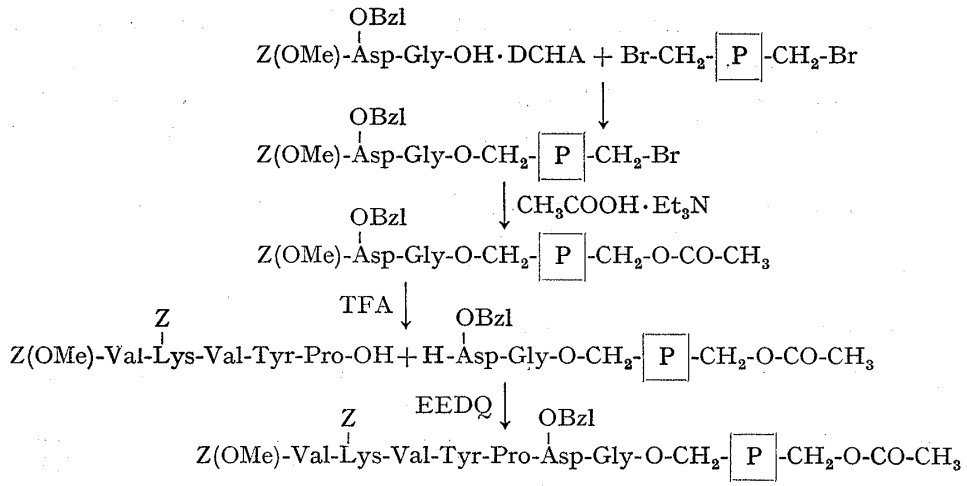

Fig. 1. Condensation of Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH with H-Asp(OBzl)-Gly-resin

rearrangement reaction of the β-benzyl-aspartyl bond[20] by the basicity of the excess DCHA. The peptide resin thus obtained did not exhibit the carbonyl absorption band typical to that of succinimide (1773 cm^{-1}) in infrared (IR) and the material recovered from the filtrate was identified with the authentic sample of Z(OMe)-Asp(OBzl)-Gly-OH·DCHA by comparison of their IR spectra and the elemental analysis. From these results, it can be concluded that Z(OMe)-Asp(OBzl)-Gly-OH was esterified onto the resin without accompanying any side reaction. Although previously we[4] used a mixture of DMF and ethanol (1:1) as a solvent, this can be substituted with DMF alone without affecting the esterification yield. Under these modified conditions, the dipeptide (II) was esterified onto the resin in nearly 52% yield. On the other hand, attempts to esterify the dipeptide (II) onto the hydroxymethylated resin[21] by DCC, carbonyldiimidazole[22] or thionyldiimidazole[23] were all unsuccessful.

After esterification of Z(OMe)-dipeptide (II) onto the resin, the unreacted bromomethyl function was converted to the acetoxymethyl group by treatment with triethylammonium acetate. In spite of this treatment, a certain amount of the innert halogen atom remained on the resin as observed previously.[4,24] The Z(OMe) group of the dipeptide anchored on the resin was cleaved with 50% TFA in methylene chloride.[25] Our preliminary tests on Z(OMe)-Lys(Z)-OH indicated that this condition minimized the decomposition of the Z group[26] and the Z(OMe) group attached at the α-amino function was completely removed within 30 min. The resin was subsequently neutralized with triethylamine. The free dipeptide resin thus obtained served to the next coupling reaction with the Z(OMe)-pentapeptide (I).

Under the following two conditions, we examined mainly the reactivity of various coupling reagents. Based on the quantity of the Z(OMe)-dipeptide on th resin, molar ratios of the carboxyl component and the reagent were settled as 2:4 and 4:8. Each reaction was performed in DMF at room temperature for 48 hr and a part of the resin was submitted for acid hydrolysis. The average yield of the coupling reaction was calculated from the recov-

20) M.A. Ondetti, A. Deer, J.T. Sheehan, J. Pluscec and O. Kocy, *Biochemistry*, **7**, 4069 (1968). See other reference thereof.
21) M. Bodanszky and J.T. Sheehan, *Chem. Ind.* (London), 1597 (1966).
22) G.W. Anderson and R. Paul, *J. Am. Chem. Soc.*, **80**, 4423 (1958).
23) H.A. Staab and K. Wendel, *Angew. Chem.*, **73**, 26 (1961); H.A. Staab and G. Walther, *Ann. Chem.*, **657**, 98 (1962).
24) B. Green and L.R. Garson, *J. Chem. Soc.* (*C*), **1969**, 401.
25) S. Karlsson, G. Lindeberg, J. Porath and U. Ragnarsson, *Acta Chem. Scand.*, **24**, 1010 (1970); B. Gutte and R.B. Merrifield, *J. Am. Chem. Soc.*, **91**, 501 (1969).
26) O.G. Nielsen and G.L. Tritsch, *Biochemistry*, **8**, 187 (1969); M. Kishi, Y. Kishida and S. Sakakibara, "Proc. of the 7th Symp. on Peptide Chemistry," Pub. Protein Research Foundation, 1969, p. 36.

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eries of Val and Pro (present in the carboxyl component) in the hydrolysate taking the recovery of Gly as the standard.

As seen in Table I, DCC gave at best 55% coupling when 4 equimoles of the carboxyl component were employed and 56% at 6 moles. Quantitative coupling by this reagent seems

Table I. Condensation Reaction of Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH with H-Asp(OBzl) Gly-Resin

Dipeptide-resin 242 mg		Z(OMe)-pentapeptide (M. W. 903)			Coupling reagent				Acid hydrolysate			Average yield of coupling
mM	ratio	mg	mM	ratio		mg	mM	ratio	1/2 Val	Pro	Gly	%
0.03	1	54.2	0.06	2	DCC	24.7	0.12	4	0.122		1.00	12.2
0.03	1	108.4	0.12	4	DCC	49.4	0.24	8	0.555	0.540	1.00	54.8
0.03	1	162.6	0.18	6	DCC	74.1	0.36	12	0.569	0.557	1.00	56.3
0.03	1	54.2	0.06	2	DCC+	24.7	0.12	4	0.436	0.397	1.00	41.7
					NHSl	13.8	0.12	4				
0.03	1	108.4	0.12	4	DCC+	49.4	0.24	8	0.505	0.433	1.00	46.9
					NHSI	27.6	0.24	8				
0.03	1	54.2	0.06	2	NEPIS	15.2	0.06	2	0.355	0.365	1.00	36.0
0.03	1	108.4	0.12	4	NEPIS	30.4	0.12	4	0.655	0.613	1.00	63.4
0.03	1	108.4	0.12	4	DCC–PCP	241.2	0.24	8	0.091		1.00	9.1
0.03	1	54.2	0.06	2	EEDQ	14.8	0.06	2	0.560	0.603	1.00	58.2
0.03	1	108.4	0.12	4	EEDQ	29.6	0.12	4	0.895	0.901	1.00	89.8

to be difficult. Further experiments indicated that DCC plus NHSI or NEPIS gave no notable improvement compared to the reaction of DCC. The complex of DCC and pentachlorophenol (PCP)[27] gave a very poor result. As mentioned earlier,[7] the coupling reaction of Asp-terminal peptides appears to be difficult. However, it is noteworthy that EEDQ gave 53% coupling when 2 moles of the Z(OMe)-pentapeptide were employed and nearly 90% coupling was achieved at 4 moles.

Next, a similar coupling reaction of Z(OMe)-pentapeptide (I) and H-Ala-Gly-resin was examined as shown in Fig. 2. The esterification of Z(OMe)-Ala-Gly-OH onto the polymer and the deblocking procedure of the protecting group were carried out in essentially the same

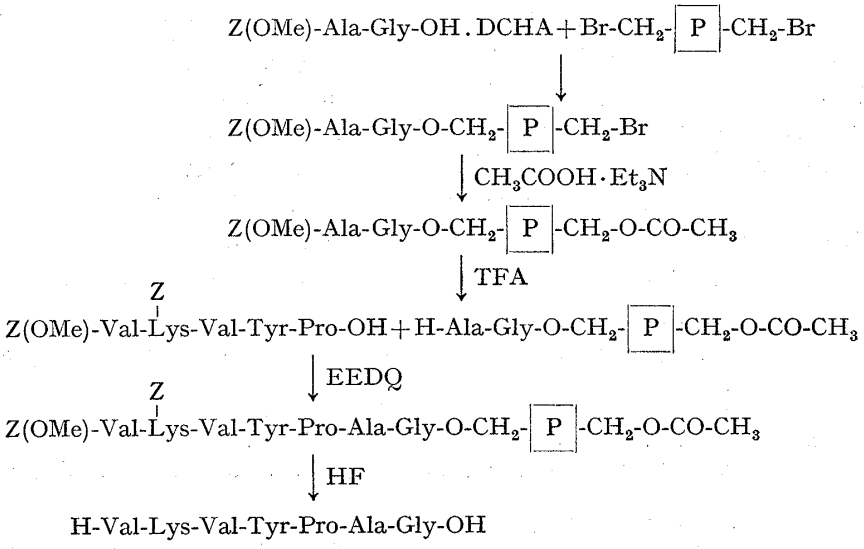

Fig. 2. Synthetic Scheme of H-Val-Lys-Val-Tyr-Pro-Ala-Gly-OH

27) J. Kovacs, L. Kisfaludy and H.Q. Ceprini, *J. Am. Chem. Soc.*, **89**, 183 (1967).

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manner as described in the above experiment. DCC gave also poor results as seen in Table II. The reaction of DCC plus NHSI improved the yield to a certain extend. This is probably due to suppression of the acyl-urea formation by NHSI. NEPIS gave 77% coupling when 4 moles of the carboxyl component were employed. The DCC-PCP complex or triphenylphosphite plus imidazole[28] gave only the yield of less than 10%. However, EEDQ gave 80% coupling at 2 moles of the carboxyl component and nearly quantitative at 4 moles.

Table II. Condensation Reaction of Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH with H-Ala-Gly-Resin

Dipeptide-resin 217 mg		Z(OMe)-pentapeptide (M. W. 903)			Coupling reagent				Acid hydrolysate			Average yield of coupling
mM	ratio	mg	mM	ratio		mg	mM	ratio	1/2 Val	Pro	Gly	%
0.03	1	54.2	0.06	2	DCC	24.7	0.12	4	0.215	0.220	1.00	21.7
0.03	1	108.4	0.12	4	DCC	49.4	0.24	8	0.474	0.447	1.00	46.0
0.03	1	54.2	0.06	2	DCC+	24.7	0.12	4	0.686	0.775	1.00	73.1
					NHSI	13.8	0.12	4				
0.03	1	108.4	0.12	4	DCC+	49.4	0.24	8	0.715	0.730	1.00	72.3
					NHSI	27.6	0.24	8				
0.03	1	54.2	0.06	2	NEPIS	15.2	0.06	2	0.385	0.420	1.00	40.3
0.03	1	108.4	0.12	4	NEPIS	30.4	0.12	4	0.735	0.800	1.00	76.8
0.03	1	108.4	0.12	4	DCC-PCP	241.2	0.12	4	0.112	—	1.00	11.2
0.03	1	54.2	0.06	2	$(C_6H_5O)_3P$	74.5	0.24	8	0.022	—	1.00	2.2
					imidazole	16.3	0.24	8				
0.03	1	54.2	0.06	2	EEDQ	14.8	0.06	2	0.895	0.815	1.00	85.5
0.03	1	108.4	0.17	4	EEDQ	29.6	0.12	4	0.975	1.02	1.00	99.8

Since, throughout these two series of experiments, EEDQ gave particularly better yield compared to the others so far examined, effective molar ratios of the carboxyl component and EEDQ were further examined using the H-Ala-Gly-resin as an amino component. The result indicated, as seen in Table III, that the coupling yield stayed around 83 to 93% when

Table III. Reactivity of EEDQ in the Coupling Reaction of Z(OMe)-Val-Lys (Z)-Val-Tyr-Pro-OH and H-Ala-Gly-Resin

Dipeptide-resin 217 mg		Z(OMe)-pentapeptide (M. W. 903)			EEDQ (M. W. 247.3)			Amino acid ratios in acid hydrolysate			Average yield of coupling
mM	ratio	mg	mM	ratio	mg	mM	ratio	1/2 Val	Pro	Gly	%
0.03	1	27.1	0.03	1	14.8	0.06	2	0.800	0.853	1.00	82.7
0.03	1	40.6	0.045	1.5	22.3	0.09	3	0.875	0.820	1.00	84.8
0.03	1	54.2	0.06	2	14.8	0.06	2	0.895	0.815	1.00	85.5
0.03	1	54.2	0.06	2	29.7	0.12	4	0.925	0.940	1.00	93.3
0.03	1	81.3	0.09	3	44.5	0.18	6	0.950	1.08	1.00	100.0

one or two moles of the Z(OMe)-pentapeptide were employed, even though in the presence of the double amount of EEDQ. Nearly quantitative coupling could only be obtained when at least 3 moles of the acid and 6 moles of EEDQ were employed. Since this condition seems to be fairly effective for this coupling reaction, a preparative experiment was carried out. The protected heptapeptide resin, Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-Ala-Gly-resin, was

28) Y.V. Mitin and O.V. Glinskaya, *Tetrahedron Letters*, **1969**, 5267.

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treated with hydrogen fluoride [29] to cleave the peptide from the resin and to remove the protecting groups, Z(OMe) and Z, at the same time. Some amount of anisole was added to prevent a possible alkylation reaction on the peptide, especially the tyrosyl residue in the molecule. The resulting heptapeptide, H-Val-Lys-Val-Tyr-Pro-Ala-Gly-OH was obtained in an analytically pure form after simple column chromatography on carboxy methyl cellulose. The over-all yield of the peptide was 46% based on the dipeptide attached on the resin.

Since EEDQ was introduced by Beallow and Malek[5] in a short communication, only limited informations about the reactivity of this reagent are available. It is proposed that the amide forming reaction by this reagent proceeds through a mixed anhydride intermediate. In order to examine a possible side reaction, *i.e.*, mainly the O-acylation, expected in the mixed anhydride, some model peptides containing the hydroxyl group were synthesized using DMF as a solvent. When the reaction of Z-Ser-OH+H-Ala-OMe, Z-Gln-OH+H-Thr-OMe and Z-Ala-OH+H-Tyr-OMe were performed in the presence of EEDQ (molar ratios of three components were 1:1:1), any other extra spots besides the product and the starting materials were detected in every case, though yields obtained were somewhat moderate than those reported in other experiments which were performed in benzene or tetrahydrofuran (THF).[5] Beallow and Malek[5] also described that the amide forming reaction with EEDQ could be carried out in a mixture of benzene and ethanol. Therefore it can be judged that the free hydroxyl group does not react with this reagent when no acid catalyst is present.[5] This reagent seems to possess, as expressed by the authors, an unique property that slow formation of and rapid consumpsion of intermediate preclude its accumulation and minimize side reactions and racemization as well. The selective activation by this reagent of carboxyl functions in the presence of other nucleophile seems to offer special advantage in the fragment condensation reaction on polymer support.

As mentioned previously,[4] in the solid phase synthesis, the excess carboxyl component and reagent can be removed from the resin by simple washing procedure, however, despite of this advantageous property, any side reaction or incomplete reaction products attached on the resin can not be removed during the synthesis and result in the formation of impure peptides in a form of unseparable mixture. Such fundamental defect of the solid phase synthesis can be overcome by the fragment condensation technique. Whether EEDQ can give satisfactory result in the coupling of other more complex peptide fragments on the polymer support remained to be examined in the future.

Experimental

General experimental methods employed are essentially the same as described in the Part XXII[30] of this series. Thin-layer chromatography was performed on silica (Kieselgel G. Merck). *Rf* values refer to the following solvent systems; Rf_1: $CHCl_3$–MeOH–H_2O (40:15:5). Rf_2: *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2).

H-Val-Tyr-Pro-OH——The title compound was prepared according to Ramachandran and Li.[17] mp 176—178°, $[\alpha]_D^{28}$ −28.5° (c=1.04 in H_2O) (lit.[17] mp 177—178°, $[\alpha]_D^{25}$ −27.4° in H_2O). *Anal.* Calcd. for $C_{19}H_{27}O_5N_3 \cdot H_2O$: C, 57.7; H, 7.4; N, 10.6. Found: C, 57.3; H, 7.3; N, 10.7.

Z(OMe)-Val-Lys(Z)-OMe——A mixed anhydride, prepared in the usual manner from Z(OMe)-Val-OH (2.81 g) in dry THF (50 ml) with triethylamine (1.5 ml) and ethyl chloroformate (1.3 ml), was added to a solution of H-Lys(Z)-OMe (prepared from 3.31 g of the hydrochloride and 1.5 ml of triethylamine) in DMF (50 ml). After the reaction mixture was stirred in an ice-bath for 2 hr, the solvent was evaporated and the residue was extracted with AcOEt, which was washed successively with 5% citric acid, 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and then evaporated. The resulting gel was purified by precipitation from AcOEt with ether; yield 3.40 g (61%), mp 140—141°. $[\alpha]_D^{25.5}$ −14.8° (c=0.95 in MeOH). *Anal.* Calcd.

29) 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. Lenard and A.B. Robinson, *J. Am. Chem. Soc.*, **89**, 181 (1967).

30) H. Yajima, Y. Okada, H. Kawatani and N. Mizokami, *Chem. Pharm. Bull.* (Tokyo), **17**, 1229 (1969).

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for $C_{29}H_{39}O_8N_3$: C, 62.5; H, 7.1; N, 7.5. Found: C, 62.6; H, 7.2; N, 7.7. The corresponding N^{α}-Boc-derivative (recrystallized from ether and petroleum ether): mp 104—105°, $[\alpha]_D^{23}$ −20.5° (c=1.02 in MeOH). *Anal.* Calcd. for $C_{24}H_{39}O_6N_5$: C, 60.8; H, 8.0; N, 8.5. Found: C, 60.9; H, 8.1; N, 8.5.

Z(OMe)-Val-Lys(Z)-NHNH$_2$——To a solution of Z(OMe)-Val-Lys(Z)-OMe (2.80 g) in MeOH (30 ml), 80% hydrazine hydrate (1.0 ml) was added and the solution was kept on standing overnight. The product formed by addition of ether was collected and recrystallized from MeOH; yield 2.50 g (90%), mp 193—195°. *Anal.* Calcd. for $C_{28}H_{39}O_7N_5$: C, 60.3; H, 7.0; N, 12.6. Found: C, 60.2; H, 7.2; N, 12.3. The corresponding N^{α}-Boc-derivative (recrystallized from MeOH and ether); mp 150—151°. *Anal.* Calcd. for $C_{24}H_{39}O_6N_5$: C, 58.4; H, 8.0; N, 14.2. Found: C, 58.2; H, 8.2; N, 14.4.

Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH (I)——To a solution of Z(OMe)-Val-Lys(Z)-NHNH$_2$ (2.79 g) in DMF (10 ml), 1N HCl in DMF (10 ml) and *t*-butylnitrite (0.65 ml) were added consecutively under cooling with ice-NaCl. After 10 min, when the hydrazine test[31] of the mixture became negative, this solution was combined to a solution of H-Val-Tyr-Pro-OH (1.89 g) in DMF (15 ml) and 1M triethylamine in DMF (10 ml). After stirring at 4° for 24 hr, the solution was condensed *in vacuo*. The residue was dissolved in AcOEt, which, after washing with 5% citric acid and H_2O, was dried over Na_2SO_4 and then evaporated. The resulting solid was recrystallized from AcOEt and ether; yield 3.22 g (71%), mp 127—129°, $[\alpha]_D^{28}$-19.7° (c=0.71 in DMF). Amino acid ratios in acid hydrolysate $Pro_{1.00}Val_{2.14}Tyr_{0.39}Lys_{0.96}$ (usually acid hydrolysis of Z-tyrosyl peptides gave low recovery of Tyr as mentioned by Iserin).[32] *Anal.* Calcd. for $C_{47}H_{62}O_{12}N_6 \cdot 1/2H_2O$: C, 61.9; H, 7.0; N, 9.2. Found: C, 61.8; H, 6.8; N, 9.3. The corresponding N^{α}-Boc-derivative (recrystallized from AcOEt): mp 142—143° $[\alpha]_D^{27}$-31.3°(c=1.05 in DMF). *Anal.* Calcd. for $C_{42}H_{62}O_{11}N_6 \cdot H_2O$: C, 60.3; H, 7.5; N, 9.8. Found: C, 60.4; H, 7.5; N, 10.0.

Z(OMe)-Asp(OBzl)-Gly-OH•DCHA Salt——A mixed anhydride prepared in the usual manner from Z(OMe)-Asp(OBzl)-OH (7.74 g) in dry THF (50 ml) with ethyl chloroformate (3.40 ml) and triethylamine (3.6 ml), was added to a solution of H-Gly-OH (2.25 g) and triethylamine (4.1 ml) in H_2O (25 ml). The solution was stirred in an ice-bath for 2 hr and then condensed *in vacuo*. The residue was acidified with 10% citric acid and the resulting precipitate was extracted with AcOEt, which, after washing with H_2O, was dried over Na_2SO_4 and then evaporated. The oily product was dissolved in acetone and DCHA (3.5 ml) was added. The white precipitate was collected by filtration and recrystallized from MeOH and ether; yield 5.61 g (45%), mp 137—141°. Rf_1 0.58 (free acid), 0.42 (DCHA salt). Amino acid ratios in acid hydrolysate $Asp_{1.09}$ $Gly_{1.00}$(average recovery 94.9%). *Anal.* Calcd. for $C_{22}H_{24}O_8N_2 \cdot C_{12}H_{23}N$: C, 65.3; H, 7.6; N, 6.7. Found: C, 65.4; H, 7.6; N, 6.7.

Z(OMe)-Ala-Gly-OH•DCHA Salt——A mixture of Z(OMe)-Ala-ONp (18.75 g), triethylamine (14 ml) and H-Gly-OH (7.5 g) in 65% aqueous dioxane (75 ml) was stirred at room temperature for 24 hr. The solvent was evaporated and the residue was dissolved in H_2O, which after washing with ether, was acidified with citric acid. The resulting precipitate was extracted with AcOEt. The organic phase was washed with H_2O, dried over Na_2SO_4 and then evaporated. The residue was dissolved in acetone and DCHA (3.5 ml) was added. The solid precipitate was recrystallized from MeOH and acetone; yield 17.10 g (70%); mp 143—144°. *Anal.* Calcd. for $C_{14}H_{18}O_6N_2 \cdot C_{12}H_{23}N$: C, 63.5; H, 8.4; N, 8.6. Found: C, 63.3; H, 8.3; N, 8.5.

Attempt to Esterify Z(OMe)-Asp(OBzl)-Gly-OH onto the Hydroxymethylated Copolymer of Styrene and 2% Divinylbenzene——The chloromethylated resin (Cl content 1.6 mM/g) was converted to the corresponding hydroxymethylated resin according to Bodanszky, *et al.*[21] This resin (125 mg) was suspended in a solution of Z(OMe)-Asp(OBzl)-Gly-OH (89 mg, 0.2 mM) in DMF (1.0 ml) and the mixture was shaken at room temperature for 48 hr in the presence of one of the following reagents; carbonyldiimidazole[22] (97 mg, 0.6 mM), thionyldiimidazole[23] (182 mg, 1 mM), DCC (42 mg, 0.2 mM) and DCC+*p*-nitrophenol+imidazole (42 mg+33 mg+136 mg). Acid hydrolysis of the every resin gave only a trace of Asp and Gly.

Z(OMe)-Asp(OBzl)-Gly-Resin——The bromomethylated copolymer of styrene and 2% divinylbenzene (4.0 g, 1.42 mM/g) was suspended in a solution of Z(OMe)-Asp(OBzl)-Gly-OH–DCHA salt (0.59 g 0.95 mM) in DMF (20 ml) and the mixture was shaken at 30° for 48 hr. The resin was collected by filtration (see below), washed successively 3 times with DMF (20 ml), EtOH (20 ml), H_2O (20 ml) and EtOH (20 ml) and then dried *in vacuo*. In the acid hydrolysate, the average content of Asp and Gly was 0.124 mM/g. Yield of the esterification step was 52%. Br content 1.10 mM/g. IR (KBr) 1720 cm^{-1} (overlapped ester carbonyl).

The material obtained from the above filtrate; 0.17 g, yield 29%, mp 138—139°, Rf_1: 0.42. Its IR spectra was identical with that of the authentic sample of Z(OMe)-Asp(OBzl)-Gly-OH·DCHA. *Anal.* Found: C, 64.8; H, 7.5; N, 6.9.

In order to examine the effects of solvent, the following two comparative reactions were performed. The bromomethylated resin (125 mg, Br 1.6 mM/g total 0.2 mM) and the equimole of Z(OMe)-dipeptide DCHA salt (125 mg, 0.2 mM) was shaken in either DMF–EtOH (1.5 ml, 1:1 v/v) or DMF (1.5 ml) at 30°

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32) B. Iserin, *Helv. Chim. Acta*, **45**, 1510 (1962).

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for 48 hr. The yield of esterification was 17.4% (average content of Asp and Gly, 0.278 mM/g) in the former solvent and 25.6% (average content of Asp and Gly 0.409 mM/g) in the latter.

Treatment of the Esterified Resin with Triethylammonium Acetate——The esterified resin obtained above (3.5 g, Br 1.10 mM/g) was shaken in DMF (15 ml) containing AcOH (0.7 ml) and Et_3N (1.6 ml) at 30° for 48 hr and then washed three times with 15 ml each of dioxane, H_2O, and MeOH. The Br content of the resin 0.39 mM/g. The resin thus obtained was used in the following coupling reaction.

Condensation of Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH with H-Asp(OBzl)-Gly-Resin——Z(OMe)-Asp (OBzl)-Gly-resin (peptide content 0.124 mM/g)(242 mg, 0.03 mM) was shaken in 50% TFA in methylene chloride (2 ml) at room temperature for 30 min. The resin was washed 3 times with 5 ml each of methylene chloride and DMF. The resin was subsequently treated with 1M Et_3N in DMF (5 ml) at room temperature for 30 min, and then washed again 3 times with DMF (5 ml each). Each coupling reaction was performed by shaking the mixture of the resin, Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH and a coupling reagent in DMF (1 ml) at 30° for 48 hr. The resin was washed 3 times with 5 ml each of DMF, MeOH, H_2O and MeOH. A part of the resin was submitted acid hydrolysis at 110° for 18 hr. Taking the recovery of Gly as the standard, the average yield of coupling was calculated from the recovery of Val and Pro. The results were listed in Table I.

Z(OMe)-Ala-Gly-Resin——A mixture of Z(OMe)-Ala-Gly-OH·DCHA (1.15 g 2.33 mM) and the bromomethylated resin (10 g, Br 1.40 mM/g) in DMF (40 ml) was shaken at 30° for 48 hr. The resin was washed as described in the preparation of Z(OMe)-Asp(OBzl)-Gly-resin. In a acid hydrolysate, the average recovery of Gly and Ala was 0.138 mM/g. Coupling yield of the dipeptide 59%. Br. content 0.79 mM/g.

The Z(OMe)-dipeptide-resin thus obtained (10 g) was shaken with AcOH (2.5 ml, 42 mM) and Et_3-N(5.9 ml, 42 mM) in dioxane (40 ml) at 30° for 48 hr. The resin was washed as described above; Br content 0.37 mM/g.

Condensation Reaction of Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH with H-Ala-Gly-Resin——In each experiment, Z(OMe)-Ala-Gly-resin (peptide content 0.138 mM/g) (217 mg 0.03 mM) was treated with 50% TFA in methylene chloride (2 ml) at room temperature for 30 min. Subsequent washing and neutralization procedure were essentially the same as described in the preparation of H-Asp(OBzl)-Gly-resin. Each coupling reaction of the Z(OMe)-pentapeptide (I) was also carried out in DMF (1 ml) at 30° for 48 hr as described. The average yield of coupling was calculated from the recoveries of Val and Pro in the acid hydrolysate, taking the recovery of Gly as the standard. The results were shown in Table II.

Reactivity of EEDQ in the Coupling Reaction of Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH and H-Ala-Gly-Resin——Starting from Z(OMe)-Ala-Gly-resin (peptide content 0.138 mM/g), H-Ala-Gly-resin was prepared as described above. Each coupling reaction was performed in DMF (1 ml) at 30° for 48 hr. The results were shown in Table III.

H-Val-Lys-Val-Tyr-Pro-Ala-Gly-OH——Z(OMe)-Ala-Gly-resin (peptide content 0.138 mM/g, 2.0 g, 0.28 mM) was treated with 50% TFA in methylene chloride (24 ml) in the presence of anisole (1 ml) at 26° for 30 min. The resin, after washing with methylene chloride and DMF (10 ml × 3 each), was treated with 1N Et_3N in DMF (25 ml) for 30 min and then washed with DMF (10 ml × 3). The resin thus obtained was suspended in DMF (20 ml) containing Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH (0.75 g, 0.84 mM) and EEDQ (0.41 g, 1.68 mM) and the mixture was shaken at 30° for 48 hr. The resin was collected by filtration, washed successively with DMF, MeOH, H_2O and MeOH (10 ml × 3) and dried over P_2O_5. Amino acid ratios in an acid hydrolysate: $Val_{1.91}Lys_{0.94}Pro_{0.91}Ala_{1.00}Gly_{1.13}$ (yield of the coupling 93%). Next the resin was treated with HF (approximately 20 ml) in the presence of anisole (1.0 ml) at 0° for 90 min. The HF was evaporated and the resin was kept on standing over KOH pellets *in vacuo* overnight. H_2O was added to the residue and the resin was removed by filtration. The filtrate was then treated with Amberlite IR-4B (acetate cycle, approximately 5 g) for 30 min. The resin was removed by filtration and the filtrate, after washing with AcOEt, was lyophilized: yield 163 mg (76% based on the amino component on the resin), Rf_2 0.48 and 0.74 (faint spot). The product was dissolved in H_2O (25 ml) and the solution, after the pH was adjusted to 6.5 with NH_4OH, was applied to a column of carboxy methyl cellulose (1.8 × 30 cm) (Serva preparation), which was eluted with H_2O (200 ml) and then with the following pyridine acetate buffer at pH 5: 0.005M (100 ml), 0.01M (400 ml), and 0.02M (250 ml). Individual fractions, 5 ml each, were collected and the absorbancy at 275 mμ was determined in each fraction. The contents of the tubes (No. 101—150) in the 0.01M eluate containing a single peak were collected and the solvent was evaporated *in vacuo*. Lyophilization of the residue gave fluffy white powder; 98 mg (46% based on the amino component on the resin), $[\alpha]_D^{28}$ −67.4° (c=1.04 in H_2O). Rf_2: 0.48. Amino acid ratios in an acid hydrolysate; $Val_{2.05}Lys_{1.03}Pro_{0.99}$ $Ala_{1.00}Gly_{0.94}$ (average recovery 88%). Amino acid ratios in AP-M digest; $Val_{1.98}Lys_{1.00}Tyr_{\fallingdotseq 0}Pro_{\fallingdotseq 0}Ala_{\fallingdotseq 0}$ $Gly_{\fallingdotseq 0}$ (complete digestion of Pro-contain peptides by AP-M seems difficult as pointed out by Jorgensen, *et. al.*).[33] *Anal.* Calcd. for $C_{35}H_{56}O_8N_9 \cdot CH_3COOH \cdot H_2O$: C, 54.8; H, 7.7; N, 13.8. Found: C, 54.4; H, 7.7; N, 13.7.

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

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Synthesis of Model Peptides——Three model peptides were synthesized. Each mixture of the carboxyl component (3.3 mM), the amino component (3.3 mM) and EEDQ (0.82 g, 3.3 mM) in DMF (8 ml) was stirred at room temperature for 48 hr. After evaporation of the solvent, the residue was dissolved in AcOEt, which was washed with 1N $NaHCO_3$, 10% citric acid and H_2O, dried over Na_2SO_4 and then evaporated. Each product was recrystallized from appropriate solvents.

Z-Ser-Ala-OMe (recrystallized from AcOEt): yield 58%, mp 112—114°, $[\alpha]_D^{26}$ −30.8° (c=1.07 in MeOH). (lit.[34] mp 113—114°). Rf_1 0.72. *Anal.* Calcd. for $C_{15}H_{20}O_6N_2$: C, 55.5; H, 6.2; N, 8.6. Found: C, 55.2; H, 6.4; N, 8.9.

Z-Glu(NH_2)-Thr-OMe (recrystallized from MeOH and AcOEt): yield 64%. mp 164—167°. $[\alpha]_D^{28}$ +1.92° (c=1.04 in DMF). Rf_1: 0.45. *Anal.* Calcd. for $C_{18}H_{25}O_7N_3$: C, 54.6; H, 6.3; N, 10.6. Found: C, 54.5; H, 6.5; N, 10.7.

Z-Ala-Tyr-OMe (recrystallized from AcOEt): yield 70%. mp 118—120°. $[\alpha]_D^{24}$ −8.77° (c=0.80 in MeOH). (lit[35] mp 121—122°). Rf_1: 0.77. *Anal.* Calcd. for $C_{21}H_{24}O_6N_2$: C, 63.0; H, 6.0; N, 7.0. Found: C, 63.3; H, 6.3; N, 6.8.

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