A Novel Synthesis of Sequential Polypeptides

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ABSTRACT: A novel method for preparing N-protected peptide *N*-hydroxysuccinimide ester as a monomer of sequential polypeptides has been developed. The method involves the synthesis of free dipeptide by the *N*-carboxyanhydride method, in the heterogeneous system of acetonitrile–water; N-protection of the free peptide by reaction with an acylated amino acid *N*-hydroxysuccinimide ester in an aqueous organic medium; and the activation of the C-terminus of the protected peptide. It has been demonstrated that the new method can yield the monomer for polycondensation more easily with full optical purity in high yields, and with fewer reaction steps than the conventional synthetic methods. The polycondensation reaction of the monomers has been also studied.

One area of recent progress in polypeptide chemistry is a successful synthesis of polypeptides with known repeating sequences of amino acids (sequential polypeptides)[2a,b] which may serve as an advanced model of native proteins. A number of the synthetic studies on these polypeptides have been published in last decade.[3-7] Most of them employed the conventional methods of peptide synthesis to obtain C-activated oligopeptides as a "monomer" for polycondensation to the sequential polypeptides. In these conventional methods, the monomer must be synthesized through many reaction steps involving a repeating cycle of the protection of amino acids, the coupling reaction of the amino acid derivatives, and the removal of the protecting group from the resulting fully protected peptide. Because of this complexity in the monomer synthesis, the sequential polypeptides cannot be obtained easily by the conventional methods and this may detract from the usefulness of the sequential polypeptides for structural studies.

Considering that the polypeptides with random sequences of amino acids are available without difficulty by copolymerization of *N*-carboxy-α-amino acid anhydrides (NCAs),[8] we have special interest in simplifing the monomer synthesis. To our knowledge, the most simplified method for the monomer synthesis is the stepwise elongation of the peptide bond employing N-protected amino acid *N*-hydroxysuccinimide (OSu) ester adopted by Katchalski *et al.*[9] and Blout *et al.*[10]

This paper describes a new synthesis of the sequential polypeptides including an improved monomer synthesis and some comparisons of the new method with the OSu method.

General Considerations on Methodological Simplification. Our method for the monomer synthesis involves three stages. First, the synthesis of the free dipeptide by the NCA method;[11] then N-protection by the condensation of the free dipeptide with a N-protected amino acid OSu ester; and finally C-activation of the N-protected peptide.

The most suitable approach for simplification of the synthetic processes leading to monomer may be to take the route involving the fewest reaction steps. The use of the NCA method for peptide synthesis, which yields directly a free dipeptide and higher oligopeptide without any protection, makes unnecessary the removal of the protecting groups of the amino acid and peptide main chain, and appears to be excellent for our approach. Although the elongation of a peptide chain having an unprotected carboxyl end by the OSu method is feasible an additional step to remove the protecting group of the N-terminus of the peptide must be added in each stage of elongation of the peptide chain. The fact that no introduction of the N-terminal protecting group is required in the elongation of peptide chain by the NCA method is an important advantage for simplification of the monomer synthesis over the OSu method. An additional advantage of the NCA method is that the NCA is a coupling reagent which can be easily prepared directly from the amino acid in high yield and stored stably in solution in acetonitrile (ACN),[12] while the OSu ester of the amino acid must be synthesized through the two steps of N-protection and OSu esterification of the amino acid, thereby lowering the overall yield.

In order to utilize the free peptide prepared by the NCA method as a monomer precursor, the steps of the N-protection of the free peptide followed by activation of the C-terminus must be performed. Direct acylation of free peptides with acyl chloride has been tried by Kopple *et al.*[13] with the unsuccessful result of undesirable oligomerization of the peptides. Therefore we chose the method of introduction of an N-protecting group in which an acylated amino acid would be reacted with a free peptide to give an acylated peptide having one amino acid residue more than the free peptide. For this purpose an acylated amino acid OSu ester[14] appears to be most suitable because the reaction proceeds in high yield in aqueous organic systems which can dissolve both the acylated ester and the free peptide. This step resembles the OSu method in which an N-protected amino acid OSu ester reacts with a free pep-

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(2) (a) D. F. DeTar, W. Honsberg, U. Honsberg, A. Wieland, M. Gouge, H. Bach, A. Tahara, W. S. Brinigar, and F. F. Rogers, Jr., *J. Amer. Chem. Soc.*, 85, 2873 (1963); (b) J. Kovacs, and A. Kapoor, *ibid.*, 87, 118 (1965).

(3) P. M. Hardy, H. N. Rydon, and H. T. Storey, *J. Chem. Soc., Parkin Trans. 1*, 1523 (1972).

(4) S. M. Bloom, S. K. Dasgupta, R. P. Patel, and E. R. Blout, *J. Amer. Chem. Soc.*, 88, 2035 (1966).

(5) A. Brack, and G. Spach, *Biopolymers*, 11, 563 (1972).

(6) M. D'Alagni, P. Bemporad, and A. Garofolo, *Polymer*, 13, 419 (1972).

(7) F. Heitz, and G. Spach, *Macromolecules*, 4, 429 (1971).

(8) C. H. Bamford, A. Elliot, and W. E. Hanby, "Synthetic Polypeptides," Academic Press, New York, N. Y., 1956.

(9) J. Ramachandran, A. Berger, and E. Katchalski, *Biopolymers*, 10, 1829 (1971).

(10) G. P. Lorenzi, B. B. Doyle, and E. R. Blout, *Biochemistry*, 10, 3046 (1971).

(11) Y. Iwakura, K. Uno, M. Oya, and R. Katakai, *Biopolymers*, 9, 1419 (1970).

(12) R. Katakai, M. Oya, K. Uno, and Y. Iwakura, *J. Org. Chem.*, 37, 327 (1972).

(13) K. D. Kopple, T. Saito, and M. Ohnishi, *J. Org. Chem.*, 34, 1631 (1969).

(14) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, 86, 1839 (1964).

tide salt resulting from the removal of the N-protecting group of the peptide derivative.[9,10]

The activation of the carboxyl group of the C-terminal amino acid residue may be achieved by converting the acylated peptide into the active ester or azide derivative. Of the active derivatives, we chose the OSu ester. Since our method and the OSu method[9,10] too must employ the step of the activation at the last stage of the synthetic scheme, we must take into account a danger of racemization of the C-terminal amino acid residue during the activation reaction. When large peptide fragments are coupled to form larger peptides by the so-called fragment condensation method with dicyclohexylcarbodiimide (DCC), the racemization of the C-terminal amino acid of the acylated peptide component can be prevented by introducing *N*-hydroxysuccinimide (HOSu).[15] So we expected to obtain the fully optically active monomer by the condensation of the acylated peptide with HOSu by DCC. The problem of racemization in introducing the activating group as well as polycondensation reaction will be discussed in a later part of this paper.

Synthesis of N-Protected Monomers. Based on the considerations mentioned above, we synthesized a number of N-protected peptide OSu esters by the NCA–OSu method. Our procedure is best illustrated by the standard preparation of benzyloxycarbonyl-L-alanyl-L-valyl-L-alanine *N*-hydroxysuccinimide ester (Z-L-Ala-L-Val-L-Ala-OSu[16]). L-Alanine as a starting amino acid[17] was allowed to react with valine NCA under well-established optimum conditions[11,18] to give pure valylalanine in 92% yield. The dipeptide was converted into an N-protected tripeptide, Z-Ala-Val-Ala-OH, by the condensation reaction with Z-Ala-OSu in ACN–water containing sodium bicarbonate[14] (80% yield) (see Scheme I).

Scheme I

$$\text{H-Ala-OH} \xrightarrow[Na_2CO_3,\ H^+]{\text{Val-NCA}} \underset{92\%}{\text{H-Val-Ala-OH}} \xrightarrow[NaHCO_3]{\text{Z-Ala-OSu}}$$

$$\underset{80\%}{\text{Z-Ala-Val-Ala-OH}} \xrightarrow{\text{HOSu, DCC}} \underset{70\%}{\text{Z-Ala-Val-Ala-OSu}}$$

One of the advantageous features of the NCA method for peptide synthesis is the rapid formation of dipeptide in quantitative yield.[11,19-21] This feature provides an advanced simplification by which the acylated tripeptide may be prepared directly form the starting amino acid without isolation and purification of an intermediate peptide. The procedure involves two successive reaction steps, that is, first, the controlled reaction of NCA with an amino acid, and, second, the condensation of the resulting dipeptide with an acylated amino acid OSu ester. Both steps can be run in the same mixed-solvent system of ACN–water, except that the presence of two different salts is necessary, the former being sodium carbonate and the latter being sodium bicarbonate. The conversion of the salt from sodium bicarbonate in the reaction of NCA, to sodium bicarbonate in the reaction of the OSu ester, is essential because the pH of the aqueous solution containing sodium carbonate is too high (pH ~11) to suppress the hydrolysis of the OSu ester. On the contrary, sodium bicarbonate has been successfully used to control the aqueous solution of the sodium salt of peptides at a suitable pH for the reaction of the OSu ester (pH ~8–9).[14] Since sodium carbonate can be fortunately converted *in situ* into sodium bicarbonate by treating the aqueous solution with hydrochloric acid of equal quantity to the sodium carbonate we have carried out the successive synthesis of Z-Ala-Val-Ala-OH from alanine.

Hydrochloric acid was added to the aqueous solution of sodium carbonate and the sodium salt of dipeptide resulting from the reaction of valine NCA with alanine. The solution of sodium bicarbonate generated *in situ* thus obtained was treated with Z-Ala-OSu in ACN (see Scheme II). After the reaction, isolation, and purification, pure Z-Ala-Val-Ala-OH could be obtained in 70% yield comparable to that (73.6%) by the procedure involving the isolation of the intermediate dipeptide. The time required for the *in situ* method of the synthesis of the acylated tripeptide was 6 hr and that for purification was 1 day. We could also synthesize Z-Ala-Met-Ala-OH in 52% yield by the *in situ* method. These results suggest that the successive *in situ* procedure is of general utility and highly advantageous for the monomer synthesis.

Scheme II

$$\text{H-Ala-OH} \xrightarrow[Na_2CO_3]{\text{Val-NCA}} \text{[H-Val-Ala-ONa]} \xrightarrow[2.\ \text{Z-Ala-OSu}]{1.\ \text{HCl}} \underset{70\%}{\text{Z-Ala-Val-Ala-OH}}$$

Activation of the carboxyl group of the acylated tripeptide was achieved by coupling with HOSu by using DCC in tetrahydrofuran.[22] The acylated monomer for polycondensation was recrystallized from isopropyl alcohol to pure product (70% yield).

Other N-protected monomers having tripeptide sequences of amino acids were also prepared by the above-described procedure. The monomers having tetrapeptide sequences were synthesized by the condensation of the acylated amino acid OSu ester with free tripeptide prepared by the NCA method followed by activation of the C-terminus. The results of the syntheses of the N-protected monomers are shown in Table I. The high overall yields of the monomers show that adoption of the controlled reaction of NCAs in the synthesis of the monomer precursor serves not only to simplify the synthetic process but also to improve the yield of the monomer.

Check of Racemization during C-Terminal Activation. As mentioned above, the active esterification of the acylated peptide with DCC contains potentially the danger of racemization at the C-terminal amino acid residue. The tendency toward racemization during the activation varies with the nature of the C-terminal residue. Since the C-terminal amino acids in this study were L-alanine and glycine, only with the former was the problem of racemization encountered. Of many methods for the check of racemization, we employed the following procedure involving the measurement of the optical purity of the amide derivatives of the acylated peptides containing C-

(15) J. E. Zimmerman, and G. W. Anderson, *J. Amer. Chem. Soc.*, 89, 7151 (1967).
(16) The nomenclature and symbols follow the Tentative Rules of the IUPAC–IUB Commission on Biochemical Nomenclature, *Biochemistry*, 5, 2485 (1966).
(17) All amino acids have the L configuration except for glycine.
(18) R. Katakai, M. Oya, K. Uno, and Y. Iwakura, *Biopolymers*, 10, 2199 (1971).
(19) R. Hirschmann, R. G. Strachan, H. Schwam, E. F. Schownewaldt, H. Joshua, B. Barkemeyer, D. F. Veber, W. J. Paleveda, Jr., T. A. Jacob, T. E. Beesley, and R. G. Denkewalter, *J. Org. Chem.* 32, 3415 (1967).
(20) R. G. Demkewalter, D. F. Veber, E. W. Holly, and R. Hirschmann, *J. Amer. Chem. Soc.*, 91, 502 (1969).
(21) D. F. Veber, R. Hirschmann, and R. G. Denkewalter, *J. Org. Chem.*, 34, 35 (1969).
(22) Tetrahydrofuran is found to be the best solvent suitable for the C-activation without racemization.

Table I
Results of Syntheses of N-Protected Monomers

N-Protected Monomer	Yield[a] (%)	Mp (°C)	[α]D in DMF	Anal. Calcd (%) C	H	N	Found (%) C	H	N
Z-Ala-Val-Ala-OSu	51.5	186–188	−33.1 (c 1.5)[b]	56.32	8.18	11.42	56.25	6.20	11.40
Z-Val-Val-Ala-OSu	50.8	194–195	−16.0 (c 1.0)	57.90	6.61	10.81	57.99	6.65	10.70
Z-Ala-Val-Val-Ala-OSu	42.3		−23.6 (c 1.0)	57.03	6.67	11.88	56.87	6.74	11.82
Z-Ala-Met-Ala-OSu	35.0	105–106	−10.3 (c 1.5)[b]	52.87	5.79	10.72	52.78	5.86	10.64
Boc-Val-Val-Gly-OSu	52.0	119–120	+14.2 (c 1.0)	53.60	7.28	11.91	53.63	7.30	11.88
Boc-Val-Gly-Val-Gly-OSu	38.2	170–172	−5.8 (c 1.0)	52.36	7.07	13.28	52.28	7.12	13.36

[a] Overall yield from the starting amino acid. [b] Measured in Me_2SO.

Table II
Polycondensation of the Monomers

Monomer Salt	Concn (g ml^{-1})	Solvent	Time (Day)	Yield (%)	η_{sp}/c[a]
HBr-H-Val-Ala-OSu	0.10	DMF	3	92	0.12
HBr-H-Ala-Val-Ala-OSu	0.20	DMF	2	100	0.22
	0.13	Me_2SO	4	98	0.18
	0.43	Me_2SO	4	100	0.23
HBr-H-Val-Val-Ala-OSu	0.43	Me_2SO	3	100	0.16
HBr-H-Ala-Val-Val-Ala-OSu	0.08	DMF	3	89	0.14
HBr-H-Ala-Met-Ala-OSu	0.60	DMF	1	100	0.20
CF_3COOH-H-Val-Gly-OSu	0.06	DMF	5	41	0.09
CF_3COOH-H-Val-Val-Gly-OSu	0.23	DMF	5	100	0.23
	0.10	DMF	5	91	0.23
CF_3COOH-H-Val-Gly-Val-Gly-OSu	0.16	DMF	5	98	0.17

[a] Measured in dichloroacetic acid at 0.5 g/100 ml at 30°.

terminal alanine, which were prepared both by our method, and by a method free from racemization (see Scheme III).

Scheme III

Z-Ala-ONp + HCl-H-Ala-OMe ⟶

Z-Ala-Ala-OMe $\xrightarrow{NH_3}$ Z-Ala-Ala-NH_2

Z-Ala-OSu + H-Ala-OH ⟶

Z-Ala-Ala-OH $\xrightarrow{HOSu,\ DCC}$

Z-Ala-Ala-OSu $\xrightarrow{NH_3}$ Z-Ala-Ala-NH_2

An acylated dipeptide methyl ester, Z-L-Ala-L-Ala-OMe, was prepared without racemization by the reaction of Z-L-Ala-ONp with HCl-H-L-Ala-OMe and converted into Z-L-Ala-L-Ala-NH_2 in 99% yield by the reaction with ammonia in methanol.[23] On the other hand, the condensation of Z-L-Ala-L-Ala-OH prepared by the OSu method with HOSu using DCC gave an acylated dipeptide active ester, Z-L-Ala-L-Ala-OSu, the C-terminal L-alanine of which might be racemized at this stage. The OSu ester was also converted into the amide derivative in 96% yield. The acylated di-L-alaninamide derived from the active ester gave the optical rotation $[\alpha]^{23}D$ of +8.30 ± 0.15° in dimethylformamide and the authentic sample obtained from the methyl ester gave +8.25 ± 0.15° in the same solvent. This result shows that the activation of the acylated peptide having C-terminal L-alanine with HOSu using DCC was practically free from racemization. The above result, together with the result that polycondensation proceeds without racemization of the OSu ester,[3] supports the fact that our method can provide a new synthetic route to sequential polypeptides with full optical activity.

Polycondensation Reaction of the Monomers. N-Protecting groups of the monomers were removed by action of hydrogen bromide or trifluoroacetic acid. The resulting monomer salts were purified by recrystallization from methanol. The monomer salts were dissolved in dimethylformamide or dimethyl sulfoxide at a concentration of 0.06 to 0.43 g ml^{-1}, and were polymerized by the addition of 1.2 equiv of triethylamine. The results of the polycondensations are summarized in Table II. The main side reaction in polycondensation of the monomer is cyclization, which can be minimized at high concentrations of the monomer.[2] Since all the monomers in this study have rather poor solubility owing to nonpolar side chains, the polymerization at the highest concentration (1.0 g ml^{-1})[2] of the monomer could not be carried out. However, even at a concentration of 0.43 g ml^{-1} the polymerization system became solid state within a few seconds after the addition of triethylamine. At a lower concentration (0.01 g ml^{-1}) the polymerization proceeded in a viscous liquid state. At all concentrations, the monomer yielded almost quantitatively the polypeptide and no appreciable amount of the cyclic by-product, except in the case of L-valylglycine-*N*-hydroxysuccinimide ester, in which a large amount of the cyclic dipeptide (diketopiperazine) and a lower yield of the polymer (41%) with low molecular weight were obtained. Since the polycondensation of L-valyl-L-alanine-*N*-hydroxysuccinimide ester gives little cyclic product, the cyclization of the monomer may depend more on the sequence of the amino acids in the monomer than on the concentration of the monomer. The tendency of a given dipeptide unit toward cyclizing to diketopiperazine may differ for various sequences of peptide OSu ester

(23) N. Izumiya and T. Yamashita, *J. Biochem.* (*Tokyo*), 46, 19 (1959).

perhaps because of the conformation of the peptide unit.[24] Clearly, the conformation of L-valylglycine-*N*-hydroxysuccinimide ester is favorable rather for cyclizing to diketopiperazine than for polymerizing to linear polymer, while that of L-valyl-L-alanine-*N*-hydroxysuccinimide ester is not. Nevertheless, we have found that the polypeptide of alternating L-valine and glycine with high molecular weight can be obtained in quantitative yield by the polycondensation of a tetrapeptide OSu ester, CF_3COOH-H-L-Val-Gly-L-Val-Gly-OSu. As an earlier study[2] has pointed out that a tetrapeptide active ester might be a better monomer for polycondensation, our result suggests that a monomer of the dipeptide unit having a tendency toward cyclizing should in general be converted into the monomer of double dipeptide unit in order to avoid the formation of the cyclic by-products.

Experimental Section

General Procedure for N-Protected Monomers. An amino acid (0.01 mol) was dissolved in an aqueous solution of 50 ml of 0.2 *N* sodium hydroxide containing 0.01 mol of sodium carbonate. After addition of 30 ml of acetonitrile (ACN) to the solution, the resulting heterogeneous solution was cooled to −10°. Addition of a 10% solution of NCA (0.012 mol) in ACN precooled to −10° to the heterogeneous solution started the controlled reaction, which was allowed with stirring for 2 hr at −10°. The ACN layer of the heterogeneous system was separated out and the aqueous layer of the system was washed twice with a 30-ml portion of cold ACN and once with 30 ml of ethyl acetate. The solution of the sodium salt of dipeptide and sodium carbonate was neutralized by dilute sulfuric acid. The resulting sodium sulfate was removed by the addition of 100 ml of ethanol followed by filtration. The alcoholic filtrate was concentrated at reduced pressure to give an oily residue. Addition of 100 ml of ethanol and 100 ml of diethyl ether crystallized the oily residue. Recrystallization of the crystals from aqueous methanol gave a pure dipeptide.

The dipeptide thus obtained (0.01 mol) was dissolved in 50 ml of 0.2 *N* sodium hydroxide containing 0.01 mol of sodium bicarbonate. To the solution was added a solution of an acylated amino acid OSu ester (0.0095 mol) in 50 ml of ACN. The reaction was allowed with stirring for 3 hr at room temperature. The heterogeneous solution became homogeneous within 5–45 min. Twice extraction of ACN from the homogeneous solution with a 50-ml portion of ethyl acetate gave an aqueous solution, which was acidified to pH 3 by addition of 2 *N* hydrochloric acid under ice cooling. An acylated peptide crystallized out after standing for 1 hr and the crystals were collected and dried. The product was dissolved in hot ethyl acetate or tetrahydrofuran if the product had poor solubility in ethyl acetate, and the solution was filtered. The filtrate was concentrated to half-volume at reduced pressure. Addition of *n*-hexane gave a pure crystalline peptide derivative.

Activation of the acylated tripeptide was carried out as follows. A solution of the acylated peptide (0.01 mol) in 100 ml of tetrahydrofuran was cooled to −5° and 1.4 g (0.012 mol) of HOSu and 2.1 g (0.011 mol) of DCC were added to the solution. The reaction system was allowed with stirring for 3 hr at −5° and for 12 hr at 5°. The system became a complete gel. Addition of a few drops of glacial acetic acid followed by evaporation of solvent at 25° gave a white solid which contained the acylated tripeptide OSu ester and dicyclohexylurea as a by-product. The solid was dissolved in hot isopropyl alcohol and the solution was filtered and cooled overnight at 0° to give a white crystals. After twice recrystallization from hot isopropyl alcohol, the product contained no dicyclohexylurea.

Acylated tetrapeptide OSu ester could be prepared by the same procedure as above. A tripeptide synthesized by the NCA method was dissolved in 0.2 *N* sodium hydroxide containing sodium bicarbonate. To the solution was added a solution of an acylated amino acid OSu ester in ACN. A part of the product crystallized out from the solution with proceeding of the reaction. After 5 hr, extraction by ethyl acetate and acidification by 2 *N* hydrochloric acid to pH 3 of the aqueous solution gave white crystals. The product was recrystallized from hot tetrahydrofuran.

The acylated tetrapeptide was dissolved in hot tetrahydrofuran and the solution was cooled to −5°. Activation was achieved by addition of HOSu and DCC in the cold solution. The active ester obtained by the same treatments as those for the tripeptide OSu ester was purified by recrystallization from hot ethanol. All the monomers synthesized by the new procedure as above are listed in Table I.

The Most Simplified "*in Situ*" Procedure for N-Protected Peptide Synthesis. Z-L-Ala-L-Val-L-Ala-OH as an Example. To the aqueous solution of sodium salt of L-valyl-L-alanine obtained by the general procedure for the peptide synthesis by the NCA method involving the reaction of 1.71 g (0.012 mol) of L-valine-*N*-carboxyanhydride with 0.89 g (0.01 mol) of L-alanine in the heterogeneous system of ACN–water was added 0.01 mol of hydrochloric acid. A solution of 3.14 g (0.0095 mol) of Z-L-Ala-OSu in 50 ml of ACN was added to the aqueous solution and the reaction was allowed with stirring for 3 hr at room temperature. The clear solution was extracted twice by a 30-ml portion of ethyl acetate and acidified by 2 *N* hydrochloric acid to pH 3 under ice cooling. Then Z-L-Ala-L-Val-L-Ala-OH crystallized out. After standing 1 hr, the crystals were filtered and dried, 2.9 g (74% overall yield). The product was dissolved in 100 ml of hot tetrahydrofuran. The solution was filtered and concentrated at reduced pressure to 40 ml at 30°. Addition of 50 ml of *n*-hexane and standing overnight at 0° gave 2.75 g (70%) of pure product.

Determination of Optical Purity of the Model Monomer. An authentic sample was synthesized without racemization as follows. The reaction of Z-L-Ala-ONp with HCl-H-L-Ala-OMe in ACN by a well-established conventional method for peptide synthesis gave Z-L-Ala-L-Ala-OMe. The acylated di-L-alanine methyl ester, 3.08 g (0.01 mol), was dissolved in 100 ml of dry methanol saturated by anhydrous ammonia and the solution was allowed to stand for 3 days at room temperature. White crystals were formed from the solution after 3 days. Evaporation of the solvent, addition of 50 ml of methanol, and evaporation again gave a white solid, which was washed with dry tetrahydrofuran and dried over P_2O_5 to yield 2.89 g (99%) of Z-L-Ala-L-Ala-NH_2.

On the other hand, Z-L-Ala-L-Ala-OSu, the C-terminal alanine of which might be racemized, was synthesized by the OSu method. A solution of 4.5 g (0.05 mol) of L-alanine was dissolved in 100 ml of 0.5 *N* sodium hydroxide containing 4 g of sodium bicarbonate. To the solution was added a solution of 15.2 g (0.0475 mol) of Z-L-Ala-OSu in 100 ml of ACN. The reaction was allowed with stirring at room temperature. After 3 hr, the solution was extracted twice by a 50-ml portion of ethyl acetate and acidified by 2 *N* hydrochloric acid under cooling. An oily product crystallized within 15 min. After standing 2 hr at 0° the crystals were separated and dried. Recrystallization from hot ethyl acetate gave 12.3 g (84% yield) of Z-L-Ala-L-Ala-OH. The acylated peptide, 2.94 g (0.01 mol), was dissolved in 50 ml of tetrahydrofuran and the solution was cooled to −5°. After addition of 1.38 g (0.012 mol) of HOSu and 2.09 g (0.011 mol) of DCC, the solution was stirred for 3 hr at −5° and an additional 12 hr at 5°. White crystals of dicyclohexylurea were filtered off and the filtrate was concentrated to give a white solid, which was dissolved in hot isopropyl alcohol. The solution was filtered and cooled to 0° overnight. Then the product crystallized was filtered and dried, 3.28 g (84% yield).

The active ester obtained above, 3.91 g (0.01 mol), was suspended to anhydrous methanol saturated by ammonia and the suspension was allowed to stand for 3 days at room temperature. The solvent was evaporated to dryness. Addition of 50 ml of methanol and evaporation were repeated twice to give finally a white solid, which was washed with water and dried over P_2O_5, 2.81 g (96% yield).

Measurement of optical rotation was carried out with a Jasco DIP-4 automatic polarimeter. Observed optical rotation of the authentic sample was $\alpha = +0.165 \pm 0.003°$ at the concentration of 5 g/100 ml of dimethylformamide (DMF) at 23°, which gave $[\alpha]^{23}D$ $+8.25 \pm 0.15°$. Observed rotation of the amide derivative from the active ester was $\alpha = +0.166 \pm 0.003°$ under the same conditions. The rotation gave $[\alpha]^{23}D$ $+8.30 \pm 0.15°$.

Polycondensation of the Monomers. Monomers, of which amino terminus was protected by the benzyloxycarbonyl group, were dissolved in 30% solution of hydrogen bromide in glacial acetic acid at the concentration of 10%. After 1 hr, diethyl ether was added to the solution to precipitate the monomer hydrobromide. The precipitate was filtered and washed repeatedly with diethyl ether and dried. The product was dissolved in the least volume of methanol and recrystallized by addition of diethyl ether. The monomers protected by *tert*-butoxycarbonyl group were dissolved in

(24) Y. Koyama and T. Shimanouchi, *Biopolymers*, 6, 1037 (1968).

trifluoroacetic acid to remove the protecting group. The same treatments as above gave the monomer trifluoroacetate.

The monomer salt was dissolved in DMF or Me_2SO at the concentration listed in Table II. To the solution was added with shaking 1.2 equiv of triethylamine. The system of high concentration of the monomer salt became immediately complete gel. In this case the polymerization was allowed without shaking. The system of lower concentration of the monomer salt kept a liquid state throughout polycondensation. After the polymerization the solid or the viscous liquid was triturated with ACN and the polymer obtained was filtered, extracted repeatedly by methanol, washed with diethyl ether, and dried.

Conformational Studies of Random DL Copolypeptides in Solution Using High-Resolution Nuclear Magnetic Resonance

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ABSTRACT: The solution conformation of several DL copolypeptides is studied using the α-CH and amide NH spectra. The coexistence of helix and coil is seen from a double-peak appearance in the spectrum. It is concluded that poly(γ-benzyl DL-glutamate) of DP $\approx$ 170 is fully helical in chloroform ($CDCl_3$) and dimethylformamide but fully coiled in dimethyl sulfoxide. Reduction of molecular weight results in the formation of a coil component in $CDCl_3$ and dimethylformamide. Poly(β-benzyl DL-aspartate) of DP $\approx$ 60 is found to be over half-random coil in $CDCl_3$ while poly(β-methyl DL-aspartate) of DP $\approx$ 140 is fully coiled in chloroform.

The solution conformations of synthetic polypeptides such as poly(γ-benzyl L-glutamate) ((Bzl-L-Glu)$_n$) have been studied mainly by the use of ORD and CD, but these techniques rest ultimately on X-ray diffraction studies of the solid state together with the assumption that the conformation is the same in both states. In the case of ((Bzl-L-Glu)$_n$), however, the solution conformation has been unambiguously determined by X-ray diffraction.[2a] ORD–CD being of limited applicability to DL copolymers, their conformations have long been the subject of study and infrared spectroscopy has to date proved the most successful technique.

Inspection of α-helical models of randomly copolymerised D and L residues shows the presence of several unfavorable nonbonded atom contacts. Despite this fact, the strong preference of a number of polyamino acids for the helical conformation in the so-called helicogenic solvents led to suggestions that such DL polypeptides might have a high proportion of helix in solution. Poly(γ-benzyl DL-glutamates) ((Bzl-L-Glu)$_n$) have been much studied and it was early shown[2b] that (Bzl-L-Glu)$_n$ in chloroform can incorporate up to 30% of the D residue without disruption of the right-handed (RH) helix. In a thorough study of (Bzl-DL-Glu)$_n$ in the solid state, using in the main the amide V vibration characteristic of helix, Tsuboi *et al.*[3] came to the conclusion that a 50 D/50 L copolymer of DP $\sim$ 150 is about one-half helical, although the helix is not the regular α, but one distorted to an unknown degree. The proportion of helix was found to rise with increase of molecular weight, although the difficulty of synthesising DL copolypeptides of very high molecular weight prevented an estimation of the limiting amount of helix possible for such a polymer. To explain these findings Tsuboi *et al.*[3] postulated a polymerization scheme based on the assumption that a growing random-coil chain shows no strong preference for addition of an L or D residue. If, however, several residues of like configuration (say L) by chance polymerize in an unbroken sequence, then a helical "seed" forms and further polymerization strongly favors addition of L monomers. Since polymerization from *N*-carboxyanhydrides proceeds from the N-terminal end, the resulting polymer has an N-terminal random-coil portion and a C-terminal helical portion. Clearly, the higher the molecular weight, the greater the helicity of the polymer.

Poly(γ-methyl DL-glutamates) ((Me-DL-Glu)$_n$) have also been studied in the solid state by Masuda *et al.*[4] by using the amide V vibration and a 50 D/50 L copolymer found to be about three-quarters helical. A recent study of the same 50 D/50 L polypeptide in films by Nakajima[5] *et al.*, who also made use of the helical amide V intensity, resulted in a value of 60% helicity for the racemic polymer. On the basis of viscosity data these authors also concluded that in solution the helical and coiled sections exist as alternating sequences rather than in the form of long blocks. Both these estimates represent the sum of right- and left-handed helices and it is clear that these racemic polymers are very considerably helical in the solid state. But what are their conformations in solution? Masuda *et al.*[4] used the hyperchromicity of the 190-mμ peptide absorption to estimate the helicity of (Me-DL-Glu)$_n$ in trifluoroethanol and concluded that the polymer was 90% helical in this solvent, *i.e.*, considerably more so than that in the solid state. This method of estimating helicity in solution is limited to solvents having the transparency at 190 mμ and the same difficulties beset the use of the amide V band in solution work. A more general method of estimating solution helicity is therefore required and high-resolution nmr can provide this.

Spach[6,7] prepared two samples of (Bzl-DL-Glu)$_n$ that differed in their hydrodynamic properties. One of these, designated B and prepared in dioxane, behaved as a rigid

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(2) (a) D. A. D. Parry and A. Elliott, *Nature (London)*, **206**, 616 (1965). (b) A. R. Downie, A. Elliott, W. E. Hanby, and B. R. Malcolm, *Proc. Roy. Soc., Ser. A*, **242**, 325 (1957).
(3) M. Tsuboi, Y. Mitsui, A. Wada, T. Miyazawa, and N. Nagashima, *Biopolymers*, **1**, 297 (1963).
(4) Y. Masuda, T. Miyazawa, and M. Goodman, *Biopolymers* **8**, 515 (1969).
(5) A. Nakajima, T. Hayashi, K. Itoh and T. Fujiwara, *Polym. J.*, **4**, 10 (1973).
(6) G. Spach, *C. R. Acad. Sci.*, **249**, 543 (1959).
(7) F. Heitz and G. Spach, *Macromolecules*, **4**, 429 (1971).