

[CONTRIBUTION FROM THE DEPARTMENT OF ORGANIC CHEMISTRY, THE HEBREW UNIVERSITY]

Acyl Chlorides of Amino Acids and Peptides as Monomers for the Preparation of Polymeric Polypeptides

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Acyl chlorides hydrochlorides of amino acids and peptides undergo polymerization, yielding polymeric polypeptides. This method is applicable in cases where N-carboxy anhydrides cannot be obtained, as with β -amino acids and peptides.

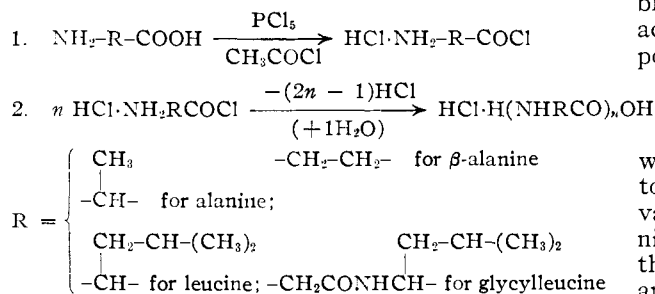
Of the various methods for the preparation of polymeric polypeptides which have been reported until recently in the literature, two permit in principle the synthesis of polypeptides in which the recurring unit represents an oligopeptide, *i.e.*, a di-, tri- or higher peptide and not an amino acid residue, namely, the condensation of esters of α -amino acids¹ and of azides of tripeptides.² Reference may also be made to two more recent papers in one of which³ the formation of chain polymers is mentioned as a side reaction in the cyclization of leucylglycylglycine, and in the other⁴ the enzymatic polymerization of several amides of dipeptides by the use of cathepsin C is reported. Moreover, up to now, no synthesis of a poly- β -amino acid has been reported in the literature. In this case, the method using an N-carboxy anhydride is not applicable.

Suitable monomers, through which polymeric peptides containing a recurring unit of at least two different α -amino acids or of a β -amino acid can be synthesized, were found in the acyl chlorides of peptides and α - or β -amino acids.⁵ These reactive substances are obtained in the form of their hydrochlorides by the action of phosphorus pentachloride on the suspension, in freshly distilled acetyl chloride, of the carefully dried and powdered and, in some cases, reprecipitated amino acid or peptide.

We have found that these acyl chlorides undergo polycondensation to a certain degree even when kept in a vacuum desiccator over phosphorus pentoxide at room temperature for some time, as indicated by a positive biuret reaction of the substance.

On heating the freshly prepared monomers in

TABLE I



(1) M. Frankel and E. Katchalski, *Nature*, **144**, 330 (1939); *THIS JOURNAL*, **64**, 2264 (1942); *ibid.*, **64**, 2268 (1942); E. Pacsu, *Nature*, **144**, 551 (1939); E. Pacsu and E. J. Wilson, *J. Org. Chem.*, **7**, 117 (1942); E. J. Wilson and E. Pacsu, *ibid.*, **7**, 126 (1942).

(2) M. Z. Magee and K. Hofmann, *THIS JOURNAL*, **71**, 1515 (1949).

(3) R. A. Boissonas and I. Schumann, *Helv. Chim. Acta*, **35**, 2229 (1952).

(4) J. S. Frinton, W. R. Hearn, V. M. Ingram, D. S. Wiggins and M. Winitz, *J. Biol. Chem.*, **204**, 891 (1953).

(5) E. Fischer, *Ber.*, **38**, 605 (1905); *ibid.*, **38**, 2914 (1905).

high vacuum or alternatively on dissolving them in dimethylformamide containing a tertiary amine, such as trimethylamine, polymers of varying chain length which are the hydrochlorides of the respective polypeptides,⁶ are produced (see Table I).

The following polymers have so far been prepared by this method: poly- β -alanine, poly-*dl*-alanine, poly-*l*-leucine and poly-(glycyl-*dl*-leucine).

Poly- β -alanine Hydrochloride.—Preparations with an average degree of polymerization of 10–14 units were obtained both by thermal condensation in high vacuum and use of trimethylamine in dimethylformamide at room temperature. The slightly yellowish crude polymers were dissolved in water and reprecipitated by absolute ethanol.

Poly-*dl*-alanine Hydrochloride.—Polymers with an average chain length of 16–18 were produced by thermal condensation. From these, by fractionation, preparations of up to 32 units, according to end-group determinations, were obtained. All these substances were water soluble and gave positive ninhydrin and biuret reactions.

Poly-*l*-leucine Hydrochloride.—Polymers prepared by the thermal method, as well as by means of trimethylamine, were composed of 20 or 10 units, respectively. They were insoluble in water or in dilute acids and slightly soluble in alkali. A positive biuret reaction was, therefore, only obtained on prolonged standing. Boiling with an aqueous solution of ninhydrin resulted in the blue coloration of the particles of the substance.

Poly-(glycyl-*dl*-leucine) Hydrochloride.—By means of thermal polymerization, preparations consisting of 4 units of the dipeptide, on the average, were obtained. These were only slightly soluble in water, but readily in dilute alkali and glacial acetic acid. Biuret and ninhydrin reactions were positive.

The chain lengths of all preparations was determined by end-group assay of both free amino nitrogen and chlorine. On prolonged heating with 6 *N* hydrochloric acid, all polymers suffered total hydrolysis, as indicated by agreement of the values found for amino nitrogen and of the total nitrogen content in the hydrolysates. Solutions of these latter were paper chromatographed and their amino acid composition compared and identified with authentic samples of the constituent amino acids.

Experimental

Micro-combustion analyses were by Drs. Weiler and Strauss, Oxford.

(6) M. Frankel, Y. Liwschitz and A. Zilkha, *Experientia*, **9**, 179 (1953).

β -Alanyl Chloride Hydrochloride.—Eight and six-tenths grams of β -alanine, reprecipitated from its aqueous solution by absolute ethanol and dried at 110°, was finely ground, driven through a hair sieve⁷ and placed in a 500-ml. glass-stoppered bottle together with 180 ml. of freshly distilled acetyl chloride. Twenty-three grams of phosphorus pentachloride was added and the mixture shaken in an ice-bath for 20 minutes. Shaking was continued for six hours more, at room temperature. Then, 10 g. of phosphorus pentachloride was added and shaking resumed for another 7.5 hours. After addition of further 6 g. of phosphorus pentachloride, shaking was continued for 6.5 hours. After that, the material was quickly filtered, washed with a small quantity of acetyl chloride and several times with dry petroleum ether and dried in a vacuum desiccator over phosphorus pentoxide. Special care was taken to exclude moisture in all operations. Twelve and nine-tenths grams of a white crystalline substance was obtained (92.5%).

Anal. Calcd. for $C_3H_7ONCl_2$: Cl, 49.2. Found: Cl, 49.0.

Polymerization of β -Alanyl Chloride Hydrochloride in High Vacuum.—Two grams of β -alanyl chloride hydrochloride was placed in the reaction vessel of a high vacuum apparatus and heated at 135° for two hours. Hydrogen chloride development virtually ceased at the end of this period. The resulting yellowish, hygroscopic material was purified by dissolving in water and reprecipitating by absolute ethanol. It gave a positive ninhydrin and bluish biuret reaction. According to determinations of free amino end-groups (Van Slyke's manometric method) and chlorine, the polymer was composed of 10 units on the average.

*Anal.*⁸ Calcd. for $C_{30}H_{53}O_{11}N_{10}Cl$: C, 47.1; H, 6.9; N, 18.3; Cl, 4.7; Van Slyke nitrogen, 1.5 (10 units). Found: C, 45.5; H, 6.9; N, 18.6; Cl, 4.7; Van Slyke nitrogen, 1.6.

Polymerization of β -Alanyl Chloride Hydrochloride by Means of Trimethylamine in Dimethylformamide.—Two grams of β -alanyl chloride hydrochloride was introduced into a 25-ml. erlenmeyer flask, with a ground-in glass stopper, and 10 ml. of a solution of trimethylamine (11%) in dimethylformamide was added. After five days, the precipitate formed, which consisted of a mixture of the polymer and trimethylamine hydrochloride, was filtered off. It was redissolved in a small quantity of water and the poly- β -alanine hydrochloride obtained by addition of absolute ethanol. It behaved like the above polymer. According to the chlorine content, the chain length was 14 on the average.

Anal. Calcd. for $C_{42}H_{73}O_{15}N_{14}Cl$: C, 48.1; H, 7.0; N, 18.7; Cl, 3.4. Found: C, 46.6; H, 6.8; N, 18.6; Cl, 2.8.

Hydrolysis of Poly- β -alanine Hydrochloride.—0.082 gram of a polymer of chain length $n = 7$ (Calcd. for $C_{21}H_{38}O_8N_7Cl$: N, 17.7. Found: N, 17.7), was boiled under reflux with 25 ml. of 6 *N* hydrochloric acid for 19 hours. The solution was then quantitatively transferred to a 100-ml. volumetric flask, neutralized with 6 *N* sodium hydroxide and made up to the mark with distilled water. Aliquot parts of the hydrolysate were used for the Van Slyke amino nitrogen determination, which yielded 17.1% (calcd. 17.7%), thus indicating complete hydrolysis of the polymer to β -alanine. Paper-partition chromatography by the ascending method, of the hydrolysate (phenol-water as mobile phase) gave the characteristic spot for β -alanine.

***dl*-Alanyl Chloride Hydrochloride.**—To 5 g. of *dl*-alanine, suspended in 100 ml. of freshly distilled acetyl chloride, 14 g. of phosphorus pentachloride was added and the mixture shaken in an ice-bath for 20 minutes. Shaking was then continued for 3 hours at room temperature. After that, the substance was quickly filtered off, washed and dried as in the case of β -alanyl chloride hydrochloride. The yield was 7.4 g. (92%).

Anal. Calcd. for $C_3H_7ONCl_2$: Cl, 49.2. Found: Cl, 48.8.

(7) This procedure, which is imperative in the case of alanine and glycine,⁶ as these amino acids do not otherwise produce satisfactory chlorides, does not, however, seem indispensable in other instances.

(8) Analyses of polymers are often not satisfactory. This holds especially for carbon (see for example W. E. Hanby, S. G. Waley and J. Watson, *J. Chem. Soc.*, 3009 (1950); C. S. Marvel, J. Dec, H. G. Cooke and J. C. Cowan, *THIS JOURNAL*, 62, 3497 (1940); K. Schloegl, F. Wessely and E. Wawersich, *Monatsh.*, 84, 705 (1953)).

Polymerization of *dl*-Alanyl Chloride Hydrochloride in High Vacuum.—Two grams of *dl*-alanyl chloride hydrochloride was polymerized by heating in high vacuum at 180° for 6 hours and a faintly yellowish water-soluble substance obtained. This was purified by dissolving in glacial acetic acid and reprecipitation by dry ether. It gave positive ninhydrin and biuret reactions. According to the free amino nitrogen content, polymers of an average chain length of 16–18 units were obtained.

Anal. Calcd. for $C_{54}H_{93}O_{19}N_{18}Cl$ ($n = 18$): C, 48.6; H, 7.0; N, 18.8; Van Slyke nitrogen, 1.05. Found: C, 44.9; H, 7.0; N, 18.7; Van Slyke nitrogen, 1.05.

Anal. Calcd. for $C_{48}H_{83}O_{17}N_{16}Cl$ ($n = 16$): C, 48.3; H, 7.0; N, 18.8; Van Slyke nitrogen, 1.25; Cl, 3.0. Found: C, 46.4; H, 7.3; N, 18.5; Van Slyke nitrogen, 1.2; Cl, 3.4; amino nitrogen after hydrolysis, 18.7.

On triturating this material with absolute ethanol, where by lower fractions were dissolved and purifying the higher fractions with glacial acetic acid-ether, polymers giving Van Slyke values of 0.66, corresponding to 32 units on the average were obtained.

***l*-Leucyl Chloride Hydrochloride.**—Five grams of *l*-leucine, suspended in 100 ml. of freshly distilled acetyl chloride, was shaken with 8 g. of phosphorus pentachloride for two hours at room temperature. After that 1.5 g. of phosphorus pentachloride was added and shaking resumed for 1.5 hours more. Filtration, washing and drying was carried out in the usual manner and 5.4 g. (76%) of the chloride was thus obtained.

Anal. Calcd. for $C_6H_{13}ONCl_2$: Cl, 38.1. Found: Cl, 38.0.

Polymerization of *l*-Leucyl Chloride Hydrochloride in High Vacuum.—Two grams of *l*-leucyl chloride hydrochloride was heated in high vacuum at 180° for 3 hours. The resulting white polymeric substance was insoluble in water or in dilute acids, but slightly soluble in alkali. On prolonged contact with the biuret reagent a positive reaction was obtained. Particles of the substance became colored blue on boiling with an aqueous ninhydrin solution. Because of the low solubility of this polymer, no Van Slyke amino nitrogen determination was made, but according to the chlorine content, it was composed of 20 units on the average.

Anal. Calcd. for $C_{120}H_{223}O_{21}N_{20}Cl$ ($n = 20$): C, 62.2; H, 9.6; N, 12.1; Cl, 1.5. Found: C, 62.1; H, 9.5; N, 11.8; Cl, 1.5; amino nitrogen after hydrolysis, 11.7.

Polymerization of *l*-Leucyl Chloride Hydrochloride by Means of Trimethylamine in Dimethylformamide.—Two grams of *l*-leucyl chloride hydrochloride was dissolved in 15 ml. of a solution of trimethylamine in dimethylformamide (11%) and kept in a glass-stoppered flask for 48 hours. The precipitate as well as the filtrate contained the polymer together with trimethylamine hydrochloride. Separation from the latter was effected by dissolving the precipitate, or the residue remaining on evaporation of the filtrate *in vacuo*, in glacial acetic acid and reprecipitating the poly-leucine hydrochloride by addition of water. Its average chain length, according to the chlorine content was 10.

Anal. Calcd. for $C_{60}H_{113}O_{11}N_{10}Cl$ ($n = 10$): C, 60.8; H, 9.5; N, 11.8; Cl, 3.0. Found: C, 60.8; H, 9.4; N, 10.7; Cl, 3.2.

Preparation of Glycyl-*dl*-leucyl Chloride Hydrochloride.—One and one-half grams of glycyl-*dl*-leucine was suspended in 30 ml. of freshly distilled acetyl chloride and on addition of 2 g. of phosphorus pentachloride the mixture was shaken for 2 hours at room temperature. During the first 1/4 hour, the dipeptide gradually dissolved, but later on crystals of glycyl-*dl*-leucyl chloride hydrochloride settled in the liquid. After the usual treatment 1.7 g. (84%) of the chloride was obtained.

Anal. Calcd. for $C_8H_{16}O_2N_2Cl_2$: Cl, 29.2. Found: Cl, 26.4.

Polymerization of Glycyl-*dl*-leucyl Chloride Hydrochloride in High Vacuum.—One gram of glycyl-*dl*-leucyl chloride hydrochloride was heated in high vacuum at 180° for 1.5 hours. The polymer thus obtained was slightly soluble in water, but readily soluble in dilute alkali and glacial acetic acid. It gave positive ninhydrin and biuret reactions and according to the chlorine content as well as free amino nitrogen

it was composed of four units of the dipeptide on the average.

Anal. Calcd. for $C_{82}H_{139}O_9N_8Cl$ ($n = 4$): C, 52.2; H, 8.0; N, 15.2; Van Slyke nitrogen, 1.92; Cl, 4.8. Found:

C, 51.5; H, 8.1; N, 14.9; Van Slyke nitrogen, 1.96; Cl, 4.5; amino nitrogen after hydrolysis, 14.5.

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The Synthesis of Peptides of L-Glutamine by the Carbobenzoxy Azide Method¹

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The carbobenzoxy azide method has been adapted to the synthesis of peptides of L-glutamine. Using this method L-glutaminyglycine, L-glutaminy-L-leucine, L-glutaminy-L-valine, L-glutaminy-L-alanine, carbobenzoxy-L-glutaminy-L-serine and carbobenzoxy-L-glutaminy-L-tyrosine have been prepared. Carbobenzoxy-L-alanyl-L-glutamine methyl ester was obtained by coupling carbobenzoxy-L-alanyl azide with L-glutamine methyl ester.

The number of glutaminy peptides that has been reported is rather small. Melville² prepared L-glutaminyglycine and L-glutaminy-L-glutamic acid by converting the carbobenzoxy-L-glutaminy derivatives to the γ -acid chlorides and treating these with ammonia. This method also has been used by Harington and Mead³ to synthesize L-glutaminy-L-cysteine and bis-(L-glutaminy)-L-cystine and by Fruton and Bergmann⁴ to prepare carbobenzoxy-L-glutaminy-L-phenylalanine. Treatment of the γ -ethyl esters of α -L-glutamic acid derivatives with ammonia was employed by Fruton and Bergmann⁴ to synthesize carbobenzoxy-L-glutaminy-L-tyrosinamide and by Miller and Waelsch⁵ to prepare L-glutaminyglycine. More recently, du Vigneaud and his co-workers⁶ reported the use of 1-tosylpyrrolid-5-one-2-carboxyl chloride in the preparation of L-glutaminy-L-asparagine.

None of these methods combines the general applicability and ease of manipulation usually encountered with peptide syntheses involving the use of the carbobenzoxy azide procedure. It therefore seemed desirable to investigate the use of this method in the synthesis of peptides of L-glutamine. The adaptation of the carbobenzoxy azide procedure depends on the success with which side reactions, introduced by the presence of the γ -amide group, can be prevented.

The starting compound for this reaction sequence, carbobenzoxy-L-glutamine methyl ester, could be prepared smoothly from the previously described carbobenzoxy-L-glutamine by reaction with diazomethane. Difficulties were expected in the conversion of this ester to the hydrazide and in the reaction of nitrous acid with the hydrazide to give the desired azide.

Esters are generally considered to show greater reactivity than amides toward hydrazine hydrate. It was hoped that this generalization would hold for

the methyl ester of carbobenzoxy-L-glutamine and that reaction conditions could be found where nearly quantitative conversion of the ester group to the hydrazide would not be accompanied by significant replacement of the γ -amide group. This goal was realized by carrying out the reaction at 0°. Using a 10% solution of the ester in methanol and excess hydrazine hydrate, the reaction appears to be complete in less than four hours and no replacement of the γ -amide group was observed.⁷

Although there are reports in the literature that the amide nitrogen of L-glutamine is abnormally susceptible to attack by nitrous acid⁸ this anomaly is apparently not observed with those derivatives in which the α -amino group is substituted.⁹ Using two molar equivalents of hydrochloric acid and equimolar amounts of sodium nitrite and carbobenzoxy-L-glutamine hydrazide, no evidence of amide hydrolysis was observed. Carbobenzoxy-L-glutaminy azide precipitates from the aqueous solution at 0° as an oil which crystallizes readily. There is therefore a very marked tendency for it to reprecipitate from solution in organic solvents since the crystalline form is less soluble than the oil. This difficulty has been overcome by filtering off the crystalline azide, washing it and drying *in vacuo*. All these operations are carried out at 0°. The azide is then dissolved in cold dimethylformamide, the amino acid ester added in ether or ethyl acetate, and the low boiling solvent is removed by evaporation. The carbobenzoxy peptide esters are precipitated, usually as crystals, by the careful addition of water. The yields in the coupling reaction varied from 55 to 81%. The conversion of the carbobenzoxy-L-glutaminy peptide esters to L-glutaminy peptides is carried out by the usual methods.

(7) In order to facilitate characterization of compounds containing both hydrazide and primary amide groups, a method was developed for the differentiation of the hydrazide and amide nitrogen. The procedure consists of acid hydrolysis of the compound followed by an iodometric titration of the liberated hydrazine (S. Siggia, "Quantitative Organic Analysis *via* Functional Groups," John Wiley and Sons, Inc., New York, N. Y., 1949, p. 75) and determination of ammonia by distillation of the residual solution. The acid hydrolysis was found to be necessary since direct iodometric titration gave low results for the hydrazine nitrogen. This is probably due to the formation of disubstituted hydrazides which can be obtained by oxidizing hydrazides with iodine (N. V. Sidgwick, "The Organic Chemistry of Nitrogen," Oxford University Press, Oxford, England, 1942, p. 398).

(8) A. C. Chibnall and R. G. Westall, *Biochem. J.*, **26**, 122 (1932).

(9) H. H. Thierfelder and E. von Cramm, *Z. physiol. Chem.*, **105**, 58 (1919).

(1) Journal Paper No. 959, New York State Agricultural Experiment Station. This investigation was supported in part by a research grant, G-3435, from the National Institutes of Health, Public Health Service. Presented in part before the Division of Organic Chemistry of the American Chemical Society, Chicago, Illinois, September 6-11, 1953.

(2) J. Melville, *Biochem. J.*, **29**, 179 (1935).

(3) C. R. Harington and T. H. Mead, *ibid.*, **30**, 1598 (1936).

(4) J. S. Fruton and M. Bergmann, *J. Biol. Chem.*, **127**, 627 (1939).

(5) H. K. Miller and H. Waelsch, *Arch. Biochem. Biophys.*, **35**, 176 (1952).

(6) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis and S. Gordon, *THIS JOURNAL*, **75**, 4879 (1953).