nitrogen and evacuated, and 50 ml of HCl was added. The tube was sealed and warmed to 25°; after 2 hr, the spectrum was recorded using a Varian A-60 nmr spectrometer. The spectrum contained a pattern of sharp absorptions between 73 and 82 cps which is characteristic of a mixture of *cis*- and *trans*-2-butene, and broad, smooth absorption between 20 and 70 cps which is attributed to alkyl proton. The ratio of integrated absorptions was 1:1.7.

**Acknowledgments.** The vpc technique was designed and all analyses were made by Mrs. Adah B. Richmond. Mr. W. M. Kipp and Mrs. Nancy Hillyard carried out the mass spectroscopic analyses and calculations. Interpretations of infrared spectra were made by Miss Naomi E. Schlichter and by Professor R. C. Lord of Massachusetts Institute of Technology.

# Polypeptides with Known Repeating Sequence of Amino Acids. Synthesis of Poly-L-glutamyl-L-alanyl-L-glutamic Acid and Polyglycylglycyl-L-phenylalanine through Pentachlorophenyl Active Ester[1]

**J. Kovacs, R. Giannotti, and A. Kapoor**

*Contribution from the Department of Chemistry, St. John's University, New York, New York.* *Received December 11, 1965*

**Abstract:** A general method for the synthesis of polypeptides with known repeating sequence of amino acids using pentachlorophenyl active esters was developed in this laboratory, and the applicability of this method is demonstrated by the synthesis of poly-Glu-Ala-Glu (XVIII) and poly-Gly-Gly-Phe (XVI). The following advantages in using active pentachlorophenyl esters were demonstrated experimentally: (a) pentachlorophenyl esters make a good combination with *t*-butyl and N-carbobenzoxy protecting groups when peptides with trifunctional amino acids are polymerized; in contrast with thiophenyl and *p*-nitrophenyl esters, the N-carbobenzoxy protecting group is selectively removed under controlled hydrogenation conditions leaving the pentachlorophenyl and *t*-butyl groups intact; (b) the resulting peptide pentachlorophenyl ester hydrochlorides as well as the intermediate active esters are easier to purify since they are frequently higher melting compounds than other active esters (Table I); (c) the very active tripeptide pentachlorophenyl esters polymerized fast in DMSO or DMF to high molecular weight polypeptides which proved to be in a practical sense (98 ± 2%) optically pure. Optically pure tripeptide active ester VII has been prepared by lengthening the peptide chain (a) from the N-terminal residue, (b) from the C-terminal residue through the active esters, and (c) by coupling of N-carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanine azide, made from hydrazide XV, with γ-*t*-butyl-L-glutamic acid pentachlorophenyl ester hydrochloride (XIII). This indicated that coupling through pentachlorophenyl esters yielded, after purification, optically pure tripeptide active esters. Highly purified HCl·H-Glu(OBu-*t*)-Ala-Glu(OBu-*t*)-OPCP (VIII) and HCl·H-Gly-Gly-Phe-OPCP (XV) gave the corresponding polypeptides XVII and XVI in good yields. Removal of the *t*-butyl protecting groups with 90% trifluoroacetic acid afforded the water-soluble polypeptide XVIII which, after extensive dialysis, gave an approximate weight average molecular weight of 25,000 and a number average molecular weight of about 16,000. The ratio of $M_w/M_n$ was 1.6, which is indicative of a not too heterogeneous polymer. The possibility of transpeptidation of α-glutamyl residues during the removal of *t*-butyl groups was investigated. Model α- and γ-tripeptides VI and XXXII were treated with trifluoroacetic acid, and it was found within 1% accuracy that transpeptidation did not occur. The free poly-Glu-Ala-Glu was not antigenic in contrast with the random polypeptide $Glu_{60}Ala_{40}$.

There has been a great demand for high molecular weight polypeptides with known repeating sequences of amino acids,[2] especially for those which are water soluble and contain trifunctional amino acids. These polypeptides would be expected to resemble proteins more closely than those consisting of only difunctional amino acids. In 1958, Katchalski, in his excellent review,[2a] summarized the work pertaining to the syntheses of such polypeptides. Practically all the methods which were developed for the stepwise synthesis of oligopeptides were tried for the synthesis of polypeptides with known repeating sequences of amino acids; however, none of these methods satisfy the various requirements for the preparation of such polymers. Since 1954 we have been working on these problems,[3] and the main difficulty in using the established coupling methods,[4] *e.g.*, mixed anhydride or dicyclohexylcarbodiimide, is the undesired side reactions which lead to early termination of polymerization. On the other hand, the thiophenyl active ester method which was used first by Wieland and Bernhard to

(1) (a) A part of this work was presented at the IUPAC Meeting, Kyoto, April 1964, and communicated by J. Kovacs and A. Kapoor, *J. Am. Chem. Soc.*, **87**, 118 (1965). (b) This is the fourth in a series of papers concerned with polypeptides with known repeating sequence of amino acids; for the previous paper in this series see J. Kovacs and B. J. Johnson, *J. Chem. Soc.*, 6777 (1965).

(2) (a) E. Katchalski and M. Sela, *Advan. Protein Chem.*, **13**, 243 (1958); (b) C. H. Bamford, H. Elliott, and W. E. Hanby, "Synthetic Polypeptides," Academic Press Inc., New York, N. Y., 1956; (c) E. R. Blout, "Polyamino Acids, Polypeptides and Proteins," M. A. Stahmann, Ed., The University of Wisconsin Press, Madison, Wis., 1962, pp 3–11; (d) P. H. Maurer, *Progr. Allergy*, **8**, 1 (1964).

(3) (a) J. Kovacs, ref 2c, pp 37–47; (b) V. Bruckner and J. Kovacs, *Acta Chim. Acad. Sci. Hung.*, **12**, 363–404 (1957), a summarizing review; (c) V. Bruckner, M. Kajtar, J. Kovacs, H. Nagy, and J. Wein, *Tetrahedron*, **2**, 211 (1958).

(4) (a) M. Goodman and G. W. Kenner, *Advan. Protein Chem.*, **12**, 465 (1957); (b) N. F. Albertson, *Org. Reactions*, **12**, 157 (1962).

prepare polyglycylvalylisoleucine (1953)[5] and later by us (1955)[6] for the synthesis of a polypeptide with a known repeating sequence containing trifunctional amino acids cannot be used for polymerization when the protecting groups are removed by catalytic hydrogenation.

Recently Wolman, *et al.*,[7] reported the polymerization of glycyl-L-seryl-L-alanine through oxidative activation of the tripepide hydrazide to yield a polymer with a molecular weight of about 3000. Vajda[8] reported the polymerization of L-glutamyl-L-histidyl-L-lysyl-L-tyrosine with DCC to give a polypeptide with a molecular weight of about 15,500 determined by the DNP technique; no other method was used to support this value. In 1963 we reported the synthesis of poly-β-L-aspartic acid through the *p*-nitrophenyl ester;[9] at the same time, DeTar[10a] independently used the *p*-nitrophenyl active ester method for the preparation of poly-β-methyl-L-aspartylglycylglycine and for a few other polymers, but the α-aspartyl polypeptide, free of methyl ester, could not be obtained. Stewart prepared several polypeptides containing glutamic acid γ-ester residues through *p*-nitrophenyl esters; no attempt was made to determine molecular weights.[10b] The *p*-nitrophenyl ester method[10c] has the same disadvantage as the thiophenyl ester in that it cannot be used in combination with catalytic hydrogenation.

Despite the disadvantages for the use of the abovementioned two active esters, we have been screening a number of other active esters with the aim of extending their scope in the synthesis of polypeptides, since it was expected that the use of an active ester would involve fewer side reactions. Obviously cyclopeptide formation could not be completely eliminated, irrespective of the activation used.

In this paper the use of pentachlorophenyl active esters[11] in the synthesis of polypeptides with known repeating sequences of amino acids as well as of the peptide intermediates is reported. This method has the following advantages.

(a) Pentachlorophenyl esters make an excellent combination with carbobenzoxy and related groups as well as with *t*-butyl protecting groups when peptides containing trifunctional amino acids are polymerized. In this combination the carbobenzoxy group can be removed selectively by catalytic hydrogenation, leaving the *t*-butyl and active ester groups intact. The C-activated peptide derivative with the free amino group so obtained is suitable for polycondensation. In addition to the pentachlorophenyl ester, any active ester group, for example, other halogenated phenyl esters such as trichlorophenyl[12] and pentafluorophenyl[13] esters, can be used and have been tried in our laboratory with success. Our selection of pentachlorophenyl esters was based on other advantages, *e.g.*, higher reactivity, higher melting points, and easy availability of the phenol component.

Systematic investigation was carried out to establish the optimal conditions for catalytic hydrogenation of pentachlorophenyl esters, since, under the normal hydrogenation conditions used for the removal of the carbobenzoxy group, some chlorine was also removed from the pentachlorophenyl residue as hydrogen chloride. When the hydrogenation was carried out in the presence of 1 mole of hydrogen chloride in absolute methanol, during 5 to 15 min of reaction time (2–3 g material with 200–300 mg of catalyst), neither halogen nor the protective *t*-butyl group was removed; the resulting active ester hydrochloride was obtained in pure form.

The use of catalytic hydrogenation for the removal of the N-protecting group is essential since the hydrogen bromide cleavage of such groups would rule out the use of *t*-butyl protecting groups. The use of *t*-butyl ester protecting groups in the preparation of aspartic and glutamic acid containing polypeptides is essential in order to avoid the extensive transpeptidation reactions caused by alkaline hydrolysis of an alkyl or a benzyl ester group.[14]

(b) Pentachlorophenyl esters of amino acids are frequently higher melting compounds than other active esters. This is demonstrated by Table I, which lists 15 carbobenzoxyamino acid pentachlorophenyl esters prepared mostly in this laboratory. For comparison, the available melting points of the corresponding 2,4,5-trichlorophenyl as well as *p*-nitrophenyl esters are also cited. The advantage of the pentachlorophenyl ester is evident when the other active esters have low melting points; *e.g.*, carbobenzoxy-α-*t*-butyl-L-glutamic acid *p*-nitrophenyl ester melts at 49–50°, and its 2,4,5-trichlorophenyl ester is an oil, while the pentachlorophenyl ester melts at 122°. Dipeptide pentachlorophenyl esters also exhibited this tendency to have higher melting points than other active esters of the same dipeptides, as indicated by compounds 16 and 17 in the table. This property of pentachlorophenyl esters helps in their purification. The high purity of the C- or N-activated peptide intermediates is essential for polymerization purposes in order to avoid undesired termination reactions.

Pentachlorophenyl esters are among the most active esters; *e.g.*, aminolysis of carbobenzoxy-L-phenylalanine pentachlorophenyl ester in dioxane with benzylamine has a half-reaction time of 1.34 min; the 2,4,5-trichlorophenyl ester gave 4.9 min and the *p*-nitrophenyl ester 23.2 min.[15] The high reactivity of pentachloro-

(5) T. Wieland and H. Bernhard, *Ann.*, **582**, 218 (1953).

(6) (a) V. Bruckner, M. Szekerke, and J. Kovacs, *Naturwiss.*, **43**, 107 (1956); (b) V. Bruckner, J. Wein, M. Kajtar, and J. Kovacs, *ibid.*, **44**, 89 (1957); (c) V. Bruckner, J. Wein, H. Nagy, M. Kajtar, and J. Kovacs, *ibid.*, **42**, 463 (1955).

(7) Y. Wolman, P. M. Gallop, A. Patchornik, and A. Berger, *J. Am. Chem. Soc.*, **84**, 1889 (1962).

(8) T. Vajda, *Chem. Ind.* (London), 785 (1963).

(9) J. Kovacs, R. Ballina, and R. Rodin, *ibid.*, 1955 (1963).

(10) (a) D. F. DeTar, *et al.*, *J. Am. Chem. Soc.*, **85**, 2873 (1963); (b) F. H. Stewart, *Australian J. Chem.*, **18**, 887 (1965); (c) M. Bodanszky, *Nature*, **175**, 685 (1955).

(11) Little attention has been given to the use of pentachlorophenyl active esters, though G. Kupryszewski (*Roczniki Chem.*, **35**, 1533 (1961); *Zeszyty Nauk, Mat. Fiz. Chem., Wyzsza Szkola Pedagog. Gdansku*, **1**, 99 (1961)) prepared a few derivatives. K. Stich and H. G. Leemann (*Helv. Chim. Acta*, **46**, 1887 (1963)) and J. Pless and R. A. Boissonnas (*ibid.*, **46**, 1609 (1963)) studied the kinetics of the aminolysis of ZPhe pentachlorophenyl ester. Preparation of a polyamino acid, poly-β-aspartic acid, and of polypeptides with known repeating sequence through tri- and pentachlorophenyl esters started in this laboratory.

(12) A few 2,4,6-trichlorophenyl esters were reported first by G. Kupryszewski and M. Kaczmarek, *Roczniki Chem.*, **35**, 935, 1533 (1961); St. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1626 (1963), made extensive use of 2,4,5-trichlorophenyl esters in peptide chemistry.

(13) B. Rakoczy, unpublished results.

(14) The *t*-butyl ester group has been proposed for the purpose of protecting aspartic and glutamic acids against transpeptidation reactions by R. Schwyzer, *et al.*, *Helv. Chim. Acta*, **44**, 1991, 2003 (1961).

(15) K. Stich and H. G. Leemann, *ibid.*, **46**, 1887 (1963).

Table I

| No. | Active ester derivative | Mp, °C $OC_6Cl_5$ | Mp, °C $OC_6H_3Cl_3$ (2,4,5) | Mp, °C $OC_6H_4NO_2$ (*p*) |
|---|---|---|---|---|
| 1 | Z-Ala | 172–173 | 104[a] | 78–79[b] |
| 2 | Z-Gly | 185[c], 134[g] | 107–108[a] | 128[d,e] |
| 3 | Z-Val | 144[f] | 94[a] | 66[e,b] |
| 4 | Z-Leu | 122–124[c] | | 95[e] |
| 5 | Z-Met | 132–133[f] | | 96–97[b] |
| 6 | Z-Ser | 192–193[g] | 137[a] | |
| 7 | Z-Phe | 158[a] | 142[a] | 126[h] |
| 8 | Z-Pro | 93–95[g] | | 94–96[i] |
| 9 | Z-Try | 170–172[f] | 115[a] | |
| 10 | Z-Cys<br>\|<br>Z-Cys | 215–217[f] | | 138–140[j] |
| 11 | Z-Glu<br>\|<br>$NH_2$ | 186–187[f] | 180[a] | 155–156[j] |
| 12 | Z-Lys<br>\|<br>Z | 154–156[f] | 113–114[a] | 58–59[b] |
| 13 | Z-Glu<br>\|<br>OBu-*t* | 94 | 107[a] | 60–61[k] |
| 14 | Z-Glu-OBu-*t* | 122–123[l] | Oil | 49–50[l] |
| 15 | Z-Asp<br>\|<br>OBZL | 151–152[m] | 92[a] | 104–105[m] |
| 16 | Z-Glu-OBu-*t*<br>\|<br>NH-$(CH_2)_2$-COOH | 151[n] | 96[n] | 56–57[n] |
| 17 | Z-Glu-OBu-*t*<br>\|<br>NH-$(CH_2)_3$-COOH | 119–120[o] | 54–55[o] | 75–75[o] |

[a] J. Pless and R. A. Boissonnas, *Helv. Chim. Acta*, **44**, 1609 (1963). [b] F. Marchiori, R. Rocchi, and E. Scoffone, *Ric. Sci., Rend.*, [6] A2, 647 (1962). [c] G. Kupryszewski and M. Formela, *Roczniki Chemi.*, **35**, 1533 (1961). [d] M. Bodanszky, *Nature*, **175**, 685 (1955). [e] B. Iselin, W. Rittel, P. Sieber, and R. Schwyzer, *Helv. Chim. Acta*, **40**, 373 (1957). [f] C. Dupraz, this laboratory. [g] M. Ceprini, this laboratory. [h] M. Bodansky and V. duVigneaud, *J. Am. Chem. Soc.*, **81**, 6074 (1959). [i] M. Bodanszky and V. duVigneaud, *ibid.*, **81**, 5688 (1959). [j] E. Schnabel, *Ann.*, **659**, 168 (1962). [k] E. Scoffone, R. Rocchi, G. Vidali, A. S Catturin, and F. Marchiori, *Gazz. Chim. Ital.*, **94**, 743 (1964). [l] G. Schmit and U. R. Ghatak, this laboratory. [m] R. Ballina and R. Rodin, this laboratory. [n] J. Kovacs and B. J. Johnson, *J. Chem. Soc.*, 6777 (1965). [o] A. Kapoor, this laboratory.

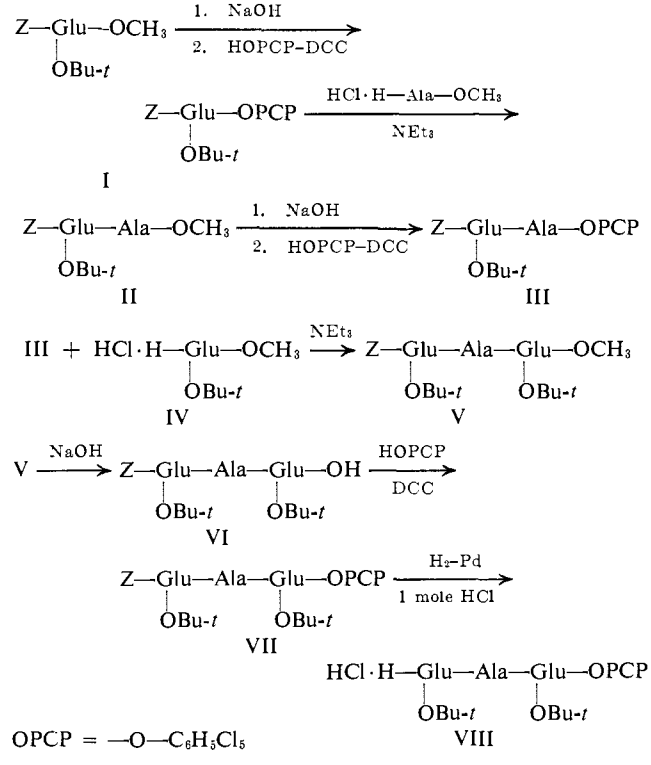
phenyl esters shortens the time for polymerization and thereby reduces possible side reactions such as aminolysis of ω-ester groups of dicarboxylic acids and imide ring formation.

Polypeptides with known repeating sequences of amino acids obtained through pentachlorophenyl esters have higher weight average molecular weights when compared with molecular weights of polypeptides with known repeating sequences of amino acids obtained by other methods. The general usefulness of the pentachlorophenyl active ester method was demonstrated by the synthesis of: (a) α-polypeptides with known repeating sequences of amino acids; (b) other than α-polypeptides, such as γ-glutamyl polypeptides; (c) polyamino acids, *e.g.*, poly-β-aspartic acid. Details of the synthesis of polypeptides of type a will be demonstrated by the preparation of poly-α-L-glutamyl-L-alanyl-L-glutamic acid and of polyglycylglycyl-L-phenylalanine.

**Synthesis of the Tripeptide Pentachlorophenyl Ester Intermediates.** For polymerization purposes a tripeptide active ester was chosen, since a dipeptide active ester would be expected to yield the diketopiperazine derivative[2] in addition to the polymer.

**Synthesis of γ-*t*-Butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic Acid Pentachlorophenyl Ester Hydrochloride (VIII).** This tripeptide active ester derivative was prepared first through pentachlorophenyl esters, lengthening the peptide chain from the N-terminal residue (method A). To prove that this purified tripeptide derivative used for polymerization was optically pure, it was synthesized through method B, that is, elongating the chain from the C-terminal residue as well as through the azide method, which is known to proceed without recemization (method C).

**Method A.** N-Carbobenzoxy-γ-*t*-butyl-L-glutamic acid pentachlorophenyl ester (I), which was obtained from N-carbobenzoxy-γ-*t*-butyl-L-glutamic acid and pentachlorophenol using the DCC method,[16] was coupled with L-alanine methyl ester to yield 72% dipeptide methyl ester II.[17] The saponified product of dipeptide II gave with pentachlorophenol and DCC N-carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanine pentachlorophenyl ester (III) in 64% yield. Coupling of dipeptide active ester III with γ-*t*-butyl-L-glutamic acid methyl ester (IV) afforded N-carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic acid methyl ester (V) in 79% yield. Saponification of tripeptide methyl ester V gave the tripeptide free acid VI in 80% yield; VI was converted into N-carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic acid pentachlorophenyl ester (VII) in 78% yield using the DCC method.[16] Tripeptide active ester VII was hydrogenated in the presence of 10% palladium-on-charcoal catalyst and one equivalent of hydrogen chloride in absolute methanol to yield γ-*t*-butyl-L-glutamyl-L-

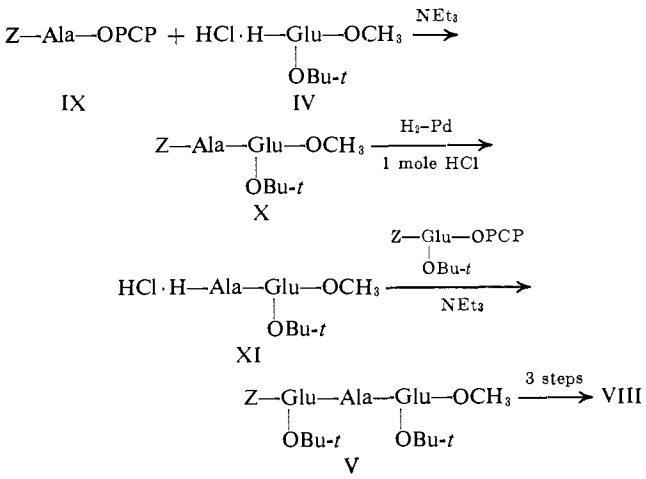



(16) D. F. Elliot and D. W. Russel, *Biochem. J.*, **66**, 49–P (1957).

(17) Abbreviations for amino acids and peptides used in this paper are those recommended in "Proceedings of the 5th European Peptide Symposium on Peptides, Oxford, Sept 1962," G. T. Young, Ed., The Macmillan Co., New York, N. Y., 1963.

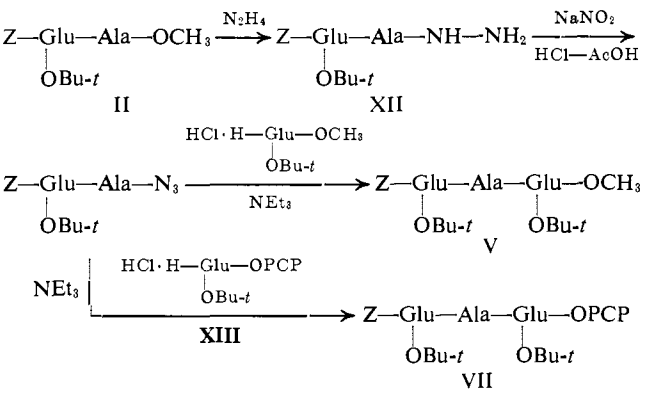
alanyl-γ-*t*-butyl-L-glutamic acid pentachlorophenyl ester hydrochloride (VIII) in 83% yield.

**Method B.** N-Carbobenzoxy-L-alanine pentachlorophenyl ester (IX), which was prepared the usual way, was coupled with γ-*t*-butyl-L-glutamic acid methyl ester (IV) in 75% yield. The resulting dipeptide methyl ester X, upon hydrogenation, gave XI, which was converted to tripeptide derivative V by coupling with pentachlorophenyl active ester I. This tripeptide derivative V was identical with that obtained through method A, including its specific rotation. The active ester derivative VIII was obtained as described under method A.




**Method C.** Since the coupling of dipeptide derivative III with IV to give tripeptide V involved the use of an active ester which might have proceeded with some racemization concerning the alanine residue, this tripeptide was also prepared through the azide coupling procedure. Thus dipeptide methyl ester II was converted to the hydrazide XII, which after conversion to the azide was coupled with IV to give tripeptide V, having identical specific rotation with compound V, prepared under methods A and B.

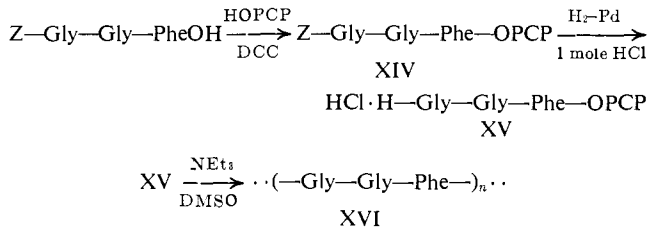
In addition the dipeptide azide obtained from XII was coupled directly to γ-*t*-butyl-L-glutamic acid pentachlorophenyl ester hydrochloride (XIII). The tripeptide pentachlorophenyl active ester VII so obtained in 46% yield was identical with that obtained through method A, including the specific rotation.

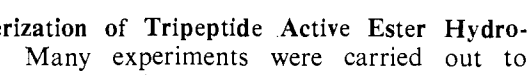



**Optical Purity of the Intermediate Peptides.** The optical purity of the active esters I and III was investigated by determining the specific rotation of the total hydrolysate and comparing that with the specific rotation of the amino acid components treated under the same conditions. In case of the glutamic acid active ester derivative I, the $[\alpha]^{30}_{565m\mu}$ for glutamic acid in the hydrolysate was +26.9° and that of the control was +26.8°. The untreated L-glutamic acid, however, gave $[\alpha]^{0}_{565m\mu}$ +33°, which indicates that under the conditions of the hydrolysis some reaction must have taken place. It is known that during acid treatment L-glutamic acid is racemized slightly,[18] and this must be one of the factors for the substantial drop of the specific rotation value in the control experiment. The fact that the [α] value for the hydrolysate was almost identical with that of the control led us to conclude that the glutamic acid residue in the active ester derivative underwent the same reactions as that in the control sample and, therefore, it was originally L in configuration. Goodman made similar observations in the case of aspartic acid peptide derivatives.[19]

The total hydrolysate of dipeptide active ester derivative III gave a $[\alpha]^{0}_{5^{\circ}5m\mu}$ for combined (Glu + Ala) of +19.7°, and that for the control was +19.76°. The $[\alpha]^{0}_{565m\mu}$ for the untreated (Glu + Ala) in the same ratio was +26.2°. Based on the same consideration discussed above, the optical purity of the dipeptide was 99.68%. However, if only the Ala residue racemized, the optical purity of Ala in the dipeptide was calculated to be 98.45%. Similar considerations allowed us to conclude that the tripeptide active ester derivative VII was practically free from racemized contaminations.

**Synthesis of N-Carbobenzoxyglycylglycyl-L-phenylalanine Pentachlorophenyl Ester (XIV).** This tripeptide active ester was prepared in 78% yield from N-carbobenzoxyglycylglycyl-L-phenylalanine and pentachlorophenol as described above. The removal of the N-protecting group from XIV by hydrogenation in the presence of one equivalent of hydrogen chloride afforded glycylglycyl-L-phenylalanine pentachlorophenyl ester hydrochloride (XV), in 80% yield.




**Polymerization of Tripeptide Active Ester Hydrochlorides.** Many experiments were carried out to establish the optimal condition for polymerization; these data will be tabulated in the next paper in this series, *i.e.*, effect of solvent, temperature, concentration, catalyst, and time. Here the method which is considered to be the most satisfactory will be mentioned.

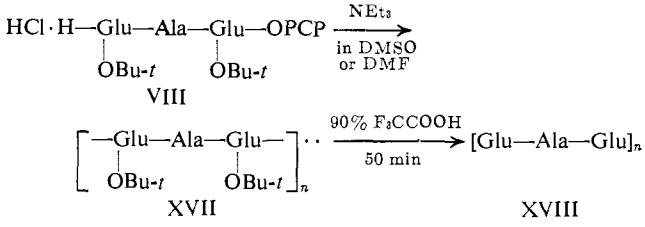
Polymerization of a tripeptide active ester containing only difunctional amino acids, such as glycylglycyl-L-phenylalanine pentachlorophenyl ester hydrochloride (XV) to polypeptide XVI proceeded smoothly and raised fewer problems than that of the tripeptides with trifunctional amino acids. The main problems are the following. It is necessary to choose a solvent which keeps the initially formed polypeptide in solution in order to obtain high molecular weight polymers. This same solvent must also allow a high concentration of the

(18) G. H. Wiltshire, *Biochem. J.*, **55**, 46 (1953).
(19) M. Goodman and F. Boardman, *J. Am. Chem. Soc.*, **85**, 2483 (1963).

tripeptide active ester to minimize cyclic peptide formation. It is essential to minimize or avoid racemization. In addition to the foregoing considerations, in the presence of trifunctional amino acids, an important problem is to eliminate or minimize undesired reactions of the side chain functional group. Specifically, here in the presence of glutamic acid, it is necessary to avoid transpeptidation reactions. These problems will be discussed in connection with the polymerization of γ-*t*-butyl-L-glutamyl-L-alanine-γ-*t*-butyl-L-glutamic acid pentachlorophenyl ester hydrochloride (VIII). This active ester was polymerized in dimethyl sulfoxide (DMSO) or dimethylformamide (DMF) solution in the presence of triethylamine; in these solvents the polymerized product remains in solution long enough to allow the formation of fairly high molecular weight polypeptides. Great care was taken to use high purity solvents as well as purified triethylamine which was completely freed from primary and secondary amines in order to avoid early termination of the polymerization reaction.[20]

To minimize cyclic peptide formation, the well-known principle of concentration factor was applied. Even if a high concentration of tripeptide active ester hydrochloride VIII is used, some cyclic peptide was expected to be present. The majority of the cyclic peptides are probably cyclohexapeptide based on Schwyzer's elegant investigations.[21] In a solvent promoting hydrogen bonding, the tripeptide esters are associated in an antiparallel fashion. In this head-to-tail arrangement the cyclohexapeptides are easily formed. This doubling reaction is very favorable in the case of peptides with odd numbers of amino acids. These considerations are important guides in designing polymerizations. Thus a tetrapeptide active ester might be a better starting material for polymerization and such investigations are under way in this laboratory. Cyclic peptides are usually more soluble in organic solvents than the polymers.[21] This property makes it possible to separate them at least partially from the polymer during the initial purification procedures. The tripeptide active ester hydrochloride VIII gave 59% poly-γ-*t*-butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic acid (XVII) after removal of the more soluble fractions. The absence of the pentachlorophenyl ester peak in the infrared spectrum at 5.58 μ is indicative of completion of the polyacylation reaction.

**Removal of the *t*-Butyl Protecting Group from the Polypeptide.** The best method to remove the *t*-butyl ester group was that reported by Schwyzer[22] using trifluoroacetic acid. The optimal conditions were established by systematic investigations[23] and found to be the use of 90% trifluoroacetic acid at room temperature for 50 min. The yield under these conditions for the free poly-L-glutamyl-L-alanyl-L-glutamic acid (XVIII), from polyester XVII, is between 79 and 95% after washing out the trifluoroacetic acid with ether. Again the completion of the reaction was established by the absence of the *t*-butyl ester peak in the infrared spectrum as well as by titration of the carboxyl groups.




In order to obtain a polymer with a narrow molecular weight distribution, an extensive dialysis was used. During this procedure it was expected that not only the small straight chain molecules would be removed, but also the small cyclic peptides. These were important considerations since the polymer was used as a protein model and was tested by Maurer for its antigenic properties.[24] The fact that the ratio of the weight average molecular weight to number average molecular weight is about 1.6 and that the polymer sedimented under a fairly uniform single peak indicates that the above-mentioned aim was approached.

**Optical Purity of the Polypeptides.** The polymerization of a tripeptide active ester involves the lengthening of the peptide chain at the carboxyl end. Therefore, racemization of the third, *i.e.*, the C-activated amino acid residue—probably through an oxazolone—should be first considered.[25] In the cases of the two examples studied here, the L-phenylalanine and one of the two L-glutamic acid residues could be involved primarily in this racemization. In contrast to the synthesis of small peptides, such as the preparation of a tripeptide from a carbobenzoxy dipeptide active ester and an amino acid ester where the racemized product might be removed by crystallization, in polycondensation of tripeptide active esters the racemized amino acid residue will be permanently incorporated in the polypeptide chain. Therefore, the optical purity is of utmost importance. The optical purity of polyglycyl-glycyl-L-phenylalanine was calculated to be 98 ± 2%, by determining the optical activity of the total hydrolysate and comparing it with that of a control. The optical purity of poly-L-glutamyl-L-alanyl-L-glutamic acid was calculated to be 99.6 ± 2%, and was determined as described for the tripeptide active ester VII. However, if only the C-terminal glutamic acid suffered racemization in the tripeptide active ester VIII during the polymerization, then the optical purity of every second glutamyl residue would be 99.2 ± 2%. In light of these results it was concluded that no significant racemization occurred during the polyacylation reactions. Presently only two coupling methods are known to proceed without racemization, the azide procedure and coupling through 1-hydroxypiperidine esters. This latter method was developed by Young.[26] How-

(20) Triethylamine was purified with carbobenzoxyglycine *p*-nitrophenyl ester and, after several days of standing at room temperature, it was considered to be free of primary and secondary amines. Two distillations gave pure triethylamine. Dimethylformamide was purified similarly.

(21) (a) R. Schwyzer, J. P. Carrion, B. Gorup, H. Nolting, and A. Tun-kyi, *Helv. Chim. Acta*, **47**, 441 (1964); (b) R. Schwyzer and A. Tun-kyi, *ibid.*, **45**, 859 (1962); (c) R. Schwyzer and P. Lieber, *ibid.*, **41**, 2168 (1958).

(22) R. Schwyzer, W. Rittel, H. Kappeler, and B. Iselin, *Angew. Chem.*, **72**, 915 (1960).

(23) Detailed investigations will be reported in the next paper in this series.

(24) P. Maurer, Seton Hall College of Medicine and Dentistry, Medical Center, Jersey City, N. J.; details of investigations will be reported elswehere.

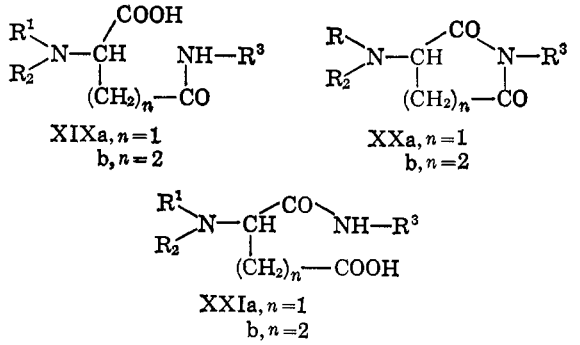
(25) M. Goodman and K. Stueben, *J. Org. Chem.*, **27**, 3409 (1962); M. Goodman and L. Levine, *J. Am. Chem. Soc.*, **86**, 2918 (1964); M. Goodman and W. J. McGahren, *ibid.*, **87**, 3028 (1965); M. W. Willams and G. T. Young, *J. Chem. Soc.*, 3701 (1964); 881 (1963).

(26) S. M. Beaumont, B. D. Handford, J. H. Jones, and G. T. Young, *Chem. Comm.* (London), 53 (1965).

ever, no satisfactory explanation is available for the exceptional behavior of these C-activated peptide derivatives. Liberek studied the base-catalyzed racemization of N-protected amino acid active esters[27] and found that 2,4,6-trichlorophenyl esters are much more resistant to racemization than thiophenyl or *p*-nitrophenyl esters. It was suggested that it is not unlikely that the steric effect exerted by the chlorine atoms in the 2 and 6 positions interferes with the loss of optical activity. This type of steric effect could be applicable here in the case of polymerization through pentachlorophenyl esters as well. Because of the general importance of racemization during coupling procedures, this problem is now being studied in detail.

**Problem of Transpeptidation Reactions.**[2a] It was essential to investigate the possibility of the intramolecular transpeptidation of the $\alpha$-glutamyl residues, which could occur during the removal of the *t*-butyl protecting groups. This would result in a polypeptide containing some $\gamma$-glutamyl residues. Intramolecular chemical transpeptidations of peptides and polypeptides with aminodicarboxylic acid residues were observed in two different cases: (1) on peptides and polypeptides containing aspartyl or glutamyl residues in the peptide chain with free $\alpha$- or $\omega$-carboxyl groups; (2) on peptides and polypeptides with esterified aspartyl and glutamyl residues under basic conditions.

(1) The formation of glutarimide derivatives XXb from N-protected $\gamma$-glutamyl dipeptides XIXb and the



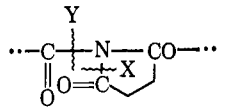
XIXa, $n=1$
b, $n=2$

XXa, $n=1$
b, $n=2$

XXIa, $n=1$
b, $n=2$

opening of the ring to yield an $\alpha$-peptide XXIb was reported first in 1952.[28] A detailed investigation of the reversible $\alpha \rightleftarrows \gamma$ transpeptidation reaction of glutamic acid peptides[2a,3,29a,b] and polypeptides[3,29c] was reported from another laboratory[30] as well.

On the other hand, formation of a five-membered ring imide, *i.e.*, 1-acyl-2-oxopyrrolidine XXII from XXIb would yield either XXIb (cleavage X) or two



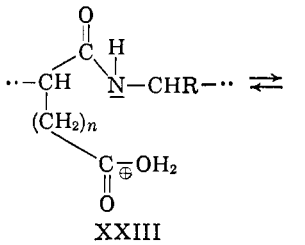
XXII

peptides, one of them with a pyroglutamyl residue at the N-terminus (cleavage Y). This latter reaction would interfere with the number average molecular weight determination through the DNP method.

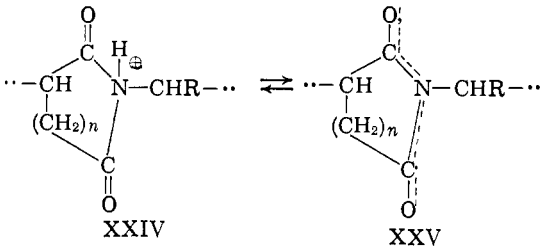
Similar observations were made on aspartic acid peptides and polypeptides where the imide ring XXa from XIXa or XXIa was obtained easily.[3,31,32] Such imide rings are formed even under conditions which are used for the hydrolysis of peptides and proteins, *i.e.*, in concentrated hydrochloric acid.[33]

(2) Transpeptidation reactions with esterified glutamyl and aspartyl derivatives and peptides were observed by Sondheimer and Holley and studied in detail by several investigators.[34] In this case the imide ring formation is a well-established base-catalyzed reaction, while formation of XXa from either XIXa or XXIa in concentrated hydrochloric acid might be considered as an acid-catalyzed reaction analogous to the acid-catalyzed esterification of carboxylic acids as indicated by XXIII, XXIV, and XXV. To prevent base-catalyzed transpeptidation reaction of esterified glutamic acid residues, Schwyzer[14] recommended the use of *t*-butyl ester as a protecting group instead of methyl or benzyl groups.

Thus during the removal of *t*-butyl ester groups from polypeptide XVII using trifluoroacetic acid, an acid-catalyzed imide ring formation through this transpepti-



XXIII



XXIV XXV

dation reaction was considered here. This possibility was investigated in detail in model compounds. N-Carbobenzoxy-$\gamma$-*t*-butyl-L-glutamyl-L-alanyl-$\gamma$-*t*-butyl-L-glutamic acid (VI) was treated with trifluoroacetic acid under the conditions used to remove *t*-butyl groups from the polypeptide. After removal of the trifluoroacetic acid the reaction product was chromatographed on paper without isolation. In several solvent systems and using large quantities (30 to 4000 $\mu$g) of the reaction

(27) B. Liberek and Z. Grzonka, *Bull. Acad. Polon. Sci., Ser. Sci. Chim.*, **12**, 367 (1964).

(28) V. Bruckner and J. Kovacs, *Magy. Tud. Akad. Kem. Tud. Oszt. Kozlemen.*, **3**, 105 (1953); presented at the Hungarian Academy of Science, Oct, 1952; a summarizing review.

(29) (a) J. Kovacs, K. Medzihradszky, and V. Bruckner, *Naturwiss.*, **41**, 450 (1954); (b) J. Kovacs, K. Medzihradszky, and V. Bruckner, *Acta Chim. Acad. Sci. Hung.*, **6**, 183 (1955); (c) V. Bruckner, J. Kovacs, and K. Medzihradszky, *Naturwiss.*, **42**, 96 (1955).

(30) (a) D. W. Clayton and G. W. Kenner, *Chem. Ind.* (London), 1205 (1953); (b) D. W. Clayton, G. W. Kenner, and R. C. Sheppard, *J. Chem. Soc.*, 371 (1956).

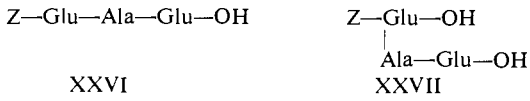
(31) (a) J. Kovacs and I. Könyves, *Naturwiss.*, **41**, 333 (1954); (b) J. Kovacs, I. Könyves, and J. Császár, *ibid.*, **41**, 575 (1954); (c) J. Kovacs and H. Mix, *ibid.*, **43**, 447 (1956).

(32) J. Kovacs, H. Nagy, I. Könyves, J. Császár, T. Vajda, and H. Mix, *J. Org. Chem.*, **26**, 1084 (1961).

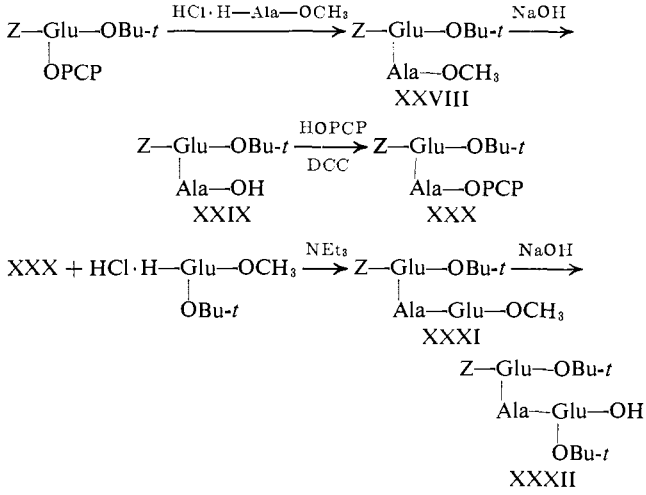
(33) I. M. Lockhart and E. P. Abraham, *Biochem. J.*, **62**, 645 (1956); M. A. Naughton, F. Sanger, B. S. Hartley, and D. C. Shaw, *ibid.*, 77, 14a (1960).

(34) (a) E. Sondheimer and R. W. Holley, *J. Am. Chem. Soc.*, **76**, 2467 (1954); E. Sondheimer and R. W. Holley, *ibid.*, **79**, 3767 (1957); (b) A. R. Battersby and J. C. Robinson, *J. Chem. Soc.*, 259 (1955); (c) L. A. Bernhard, A. Berger, J. H. Carter, E. Katchalski, M. Sela, and Y. Shalitin, *J. Am. Chem. Soc.*, **84**, 2421 (1962); (d) A. J. Adler, G. D. Fasman, and E. R. Blout, *ibid.*, **85**, 90 (1963).

product, only one spot was detected using bromphenol blue[35] indicator. If transpeptidation had occurred, in



addition to the $\alpha$-tripeptide XXVI, the $\gamma$-tripeptide XXVII would have been present also and was expected to be detectable on the chromatogram up to 1%. However, the possibility of identical or nearly identical $R_f$ values for the $\alpha$ and $\gamma$ isomers was not completely excluded and, therefore, the $\gamma$-tripeptide was synthesized. The synthesis of N-carbobenzoxy-$\alpha$-*t*-butyl-L-glutamyl-L-alanyl-$\gamma$-*t*-butyl-L-glutamic acid (XXXII) is outlined below.



The $\gamma$-tripeptide XXXII was prepared by utilizing the pentachlorophenyl ester method again. In the case of the synthesis of the $\alpha$-tripeptide through the pentachlorophenyl ester, it was demonstrated that the purified $\alpha$-tripeptide VII was practically free of racemized by-product. Therefore, no attempt was made to prove the optical purity of XXXII.

Removal of the *t*-butyl ester groups was accomplished under the conditions used for the $\alpha$ isomer and the reaction product was chromatographed on paper without isolation. In most of the solvent systems the $R_f$ values were identical with that of the $\alpha$ isomer. Two solvent systems, however, that is, pyridine–water and phenol–citric acid–disodium phosphate buffer (pH 7.6), were satisfactory, and the $R_f$ values were found to be 0.79, 0.79 and 0.68, 0.62 for the $\alpha$- and $\gamma$-tripeptides XXVI and XXVII, respectively. The $\alpha$- and $\gamma$-tripeptides XXVI and XXVII separated well even when their mixture was chromatographed in these solvent systems.

Since it was possible to detect 1% of either of the tripeptides in the presence of the other one, the $\alpha$-(XXVI) and the $\gamma$-tripeptides (XXVII) were considered to be at least 99% pure. These results indicate that practically no transpeptidation reaction and also no peptide bond cleavage occurred during the removal of the *t*-butyl ester groups with our model compounds. If we apply these results for the polypeptide *t*-butyl ester XVII, it is safely assumed that during the removal of the *t*-butyl ester groups by 90% trifluoroacetic acid transpeptidation reaction practically did not occur and so the poly-L-glutamyl-L-alanyl-L-glutamic acid (XVIII) is at least a 99% pure $\alpha$-polypeptide.

**Molecular Weight Determinations. Number average molecular weight** was determined by amino end-group analysis using Sanger's DNP method.[36] A sample of poly-Glu-Ala-Glu, which was purified by extensive dialysis and had an intrinsic viscosity value of 0.308 dl/g in dichloroacetic acid, was converted into the DNP–polypeptide. In order to evaluate the number average molecular weight, two procedures were used: (1) direct measurement of the absorbance at 360 m$\mu$ of the DNP–polypeptide gave a molecular weight of 16,400; (2) after the total hydrolysis of the DNP–polypeptide the DNP–glutamic acid was isolated and its amount was determined again by measuring the absorbance at 360 m$\mu$. The number average molecular weight so obtained was 14,400. Control experiments with DNP–glutamic acid indicated a loss of 3.5 to 5.5% under the conditions used for the total hydrolysis.

**Weight Average Molecular Weights.** Weight average molecular weight was determined in the ultracentrifuge by the sedimentation velocity method. The measurements were made at a concentration of 1.1% in 0.5 *M* Tris buffer solution at pH 7.6 at a speed of 59,780 rpm, and no attempts were made to extrapolate to infinite dilution. The values, therefore, are considered to be apparent molecular weights. The polymer sedimented under a single uniform peak which broadened gradually over a period of 96 min. Sedimentation and diffusion values were $s_{20w}^{app} = 1$ S, and $D_{20w}^{app} = 6.49 \times 10^{-7}$ cm$^2$/sec. From these values the molecular weight of approximately 25,000 was calculated[37] using an assumed partial specific volume of 0.72. The calculated $M_w/M_n$ ratio of 1.66 would indicate the not too heterogeneous nature of the polymer, which is expected after extensive dialysis used for the purification of the polymer.

**Biological Investigation.**[24] Poly-Glu-Ala-Glu was tested for antigenicity in rabbits and guinea pigs employing several immunizations with complete Freund's adjuvant. In addition the technique for forming an aggregate with methylated bovine serum albumin was employed. The analysis of the sera and the cutaneous reaction of guinea pigs indicated no detectable antibodies. In contrast with these results, the random copolymer $Glu_{60}Ala_{40}$ was antigenic in these tests. These results will be reported by Dr. P. Maurer in detail elsewhere.

## Experimental Section

The microanalyses were carried out by Drs. G. Weiler and F. B. Strauss, Oxford, England. All melting points are uncorrected and were taken on a Mel-Temp apparatus. Infrared spectra were made in potassium bromide pellets. All optical rotations were measured with a Rudolph precision ultraviolet polarimeter, Model 200S-340-80Q3, using a mercury (Type 5H Hanovia) quartz lamp and sodium lamp.

**Preparation of the Tripeptide Pentachlorophenyl Ester VIII According to Method A. N-Carbobenzoxy-$\gamma$-*t*-butyl-L-glutamic Acid $\alpha$-Pentachlorophenyl Ester (I).** To a solution of N-carbobenzoxy-$\gamma$-*t*-butyl-L-glutamic acid methyl ester[38] (8 g, 0.0228 mole) in acetone (40 ml), sodium hydroxide (1 g, 0.025 mole) in water

(35) H. J. Petrowitz and G. Pastuska, *J. Chromatog.*, 7, 128 (1962).

(36) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(37) The ultracentrifuge studies were carried out at Boyce Thompson Institute, Yonkers, N. Y., and we gratefully acknowledge the helpful discussions and the interpretations of the results by Dr. R. Heimer of the Seton Hall College of Medicine, Jersey City, N. J., and Dr. R. Staples of Boyce Thompson Institute.

(38) E. Klieger and H. Gibian, *Ann.*, **655**, 201 (1962).

(30 ml) was added. The mixture was stirred at room temperature for 1 hr, acetone was removed under reduced pressure, and the unsaponified material was extracted with ether (two 30-ml portions). The aqueous alkaline solution was acidified with dilute hydrochloric acid and rapidly extracted with ethyl acetate (two 100-ml portions). Ethyl acetate was washed with water (two 50-ml portions) and dried with sodium sulfate, and the solvent was removed under vacuum to afford N-carbobenzoxy-L-glutamic acid γ-*t*-butyl ester (6.5 g, 84.6%) as a colorless, viscous oil. This oil (6.5 g, 0.0193 mole) was dissolved in dry methylene chloride (80 ml), and dicyclohexylcarbodiimide (DCC) (4.6 g, 0.022 mole) was added to the solution. The mixture was stirred at 0–5° for 15 min, then pentachlorophenol (5.6 g, 0.021 mole) was added to it. Stirring was continued for 3 hr at 0–5°, and then overnight at room temperature. The excess DCC was decomposed with glacial acetic acid (3.5 ml). The precipitate was filtered off and the filtrate was concentrated under vacuum to give a semisolid material which was triturated with ethyl acetate (150 ml). The insoluble urea was removed by filtration. This procedure was repeated twice. The ethyl acetate solution was washed with 1 *N* hydrochloric acid (two 40-ml portions), water (40 ml), 5% aqueous sodium bicarbonate (40 ml), and water (three 50-ml portions). After drying the ethyl acetate layer over sodium sulfate and removal of the solvent under reduced pressure, a white solid was obtained which was crystallized from methanol, to yield 8 g (71.0%) of the active ester I, mp 94–95°. Recrystallization from the same solvent did not change the melting point, $[\alpha]^{25}D$ −12.0° (*c* 3.38, chloroform). The infrared spectrum had a peak at 5.6 μ characteristic of pentachlorophenyl esters.

*Anal.* Calcd for $C_{23}H_{22}NO_6Cl_5$: C, 47.18; H, 3.76; N, 2.39. Found: C, 47.14; H, 3.75; N, 2.36.

**N-Carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanine Methyl Ester (II).** To a solution of pentachlorophenyl ester I (6 g, 0.0102 mole) in dry methylene chloride (50 ml), a mixture of L-alanine methyl ester hydrochloride (1.6 g, 0.0115 mole) and triethylamine (1.6 ml, 0.0115 mole) in methylene chloride (10 ml) was added with stirring. 2-Hydroxypyridine[39] (0.150 g) was added to the above mixture and the stirring was continued for 24 hr at room temperature. The mixture was filtered from triethylamine hydrochloride and the filtrate was evaporated under vacuum. The residue was taken up in ethyl acetate (100 ml) and the insoluble triethylamine hydrochloride was filtered. The filtrate was extracted three times with 30-ml portions of 1 *N* hydrochloric acid, water, 5% aqueous sodium bicarbonate, and water. The ethyl acetate solution was dried over sodium sulfate and evaporated under reduced pressure. The residual oil was crystallized from ether–petroleum ether (bp 30–60°) to yield 3.1 g of the dipeptide II (72%), mp 97–98°. Two more recrystallizations from the same solvents raised the melting point to 98°, $[\alpha]^{25}D$ −6.7° (*c* 3.24, chloroform). The infrared spectrum showed peaks at 6.05 (amide I) and 6.5 μ (amide II).

*Anal.* Calcd for $C_{21}H_{30}N_2O_7$: C, 59.60; H, 7.10; N, 6.65. Found: C, 59.60; H, 7.18; N, 6.77.

**N-Carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanine Pentachlorophenyl Ester (III).** Dipeptide methyl ester II (3 g, 0.0071 mole) was saponified as described previously and was converted to dipeptide active ester III in 64% yield after crystallization from methanol, mp 171–172°. Three recrystallizations from the same solvent raised the melting point to 173–174°, $[\alpha]^{25}D$ −18.5° (*c* 1.33, chloroform).

*Anal.* Calcd for $C_{26}H_{27}N_2O_7Cl_5$: C, 47.50; H, 4.12; N, 4.27. Found: C, 47.44; H, 4.06; N, 4.54.

**γ-*t*-Butyl-L-glutamic Acid Methyl Ester Hydrochloride (IV).** Palladium–charcoal catalyst (10%, 0.5 g), suspended in glacial acetic acid (1 ml) and methanol (20 ml), was hydrogenated at atmospheric pressure. Dry methanol containing 0.150 g of hydrogen chloride per milliliter (0.29 ml, 0.012 mole) was added to the hydrogenated catalyst, and the hydrogenation was continued until no further uptake of hydrogen occurred. A solution of N-carbobenzoxy-γ-*t*-butyl-L-glutamic acid methyl ester (4 g, 0.0114 mole) in methanol (10 ml) was added to the reaction mixture and the hydrogenation was carried out until no further uptake of hydrogen occurred (110 ml in 7 min). The reaction mixture was filtered from the catalyst and the filtrate was concentrated under reduced pressure to a small volume. Ether (50 ml) was added to precipitate the hydrochloride, 2.55 g (88%), mp 125–126°, $[\alpha]^{26}D$ +23.7° (*c* 1, methanol) (lit.[38] mp 125–126°, $[\alpha]^{25}D$ +23.9° (*c* 1.02, methanol)).

**N-Carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic Acid Methyl Ester (V).** Dipeptide active ester III (3 g, 0.00457 mole) was coupled with diester hydrochloride IV in the usual manner to afford 79% of tripeptide methyl ester V, mp 112–113°. Two recrystallizations from ether–petroleum ether raised the melting point to 114–115°, $[\alpha]^{25}D$ −12.5° (*c* 3, chloroform).

*Anal.* Calcd for $C_{31}H_{45}N_3O_{10}$: C, 59.20; H, 7.40; N, 6.91. Found: C, 59.41; H, 7.36; N, 6.56.

**N-Carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic Acid (VI).** Tripeptide methyl ester V (0.607 g, 0.001 mole) was saponified using the procedure described previously and the tripeptide free acid VI was crystallized from ether–petroleum ether in 80% yield, mp 57–59°. This was chromatographed on silica gel; the chloroform eluate (110 ml) afforded the pure sample, which after crystallization from ether–petroleum ether melted at 58–59°, $[\alpha]^{25}D$ +3.03° (*c* 3, chloroform).

*Anal.* Calcd for $C_{25}H_{43}N_3O_{10}$: C, 58.71; H, 7.25; N, 7.09. Found: C, 58.74; H, 7.18; N, 7.13.

**N-Carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic Acid Pentachlorophenyl Ester (VII).** Tripeptide free acid VI (5.93 g, 0.01 mole) was converted to tripeptide active ester VII, using the usual method, and the resulting white solid was crystallized from ethyl acetate–ether–petroleum ether, in 78% yield, mp 132–133°. Further recrystallization from the same solvents did not raise the melting point. However, when it was recrystallized from methanol, a higher melting form, mp 151–152°, was obtained. Recrystallization of the methanol-crystallized product from ethyl acetate–ether–petroleum ether gave back the low-melting form: mp 132–133°, $[\alpha]^{25}D$ −13.2° (*c* 2, chloroform), for the low-melting form, and −13.25° (*c* 2, chloroform) for the high-melting form. The infrared spectra for both the low- and the high-melting forms were identical.

*Anal.* Calcd for $C_{35}H_{42}N_3O_{10}Cl_5$: C, 49.95; H, 4.99; N, 4.99. Found: C, 49.43; H, 5.04; N, 4.90 (high-melting form, mp 151–152°); C, 49.76; H, 5.18; N, 5.03 (low-melting form, mp 132–133°).

**γ-*t*-Butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic Acid Pentachlorophenyl Ester Hydrochloride (VIII).** Tripeptide active ester VII (0.841 g, 0.001 mole) was hydrogenated as described previously and tripeptide active ester hydrochloride VIII was isolated in 83% yield, upon crystallization from methanol–ether, mp 155–156°, $[\alpha]^{25}D$ −32.7° (*c* 3.7, methanol).

*Anal.* Calcd for $C_{27}H_{37}N_3O_8Cl_6$: C, 43.55; H, 4.97; N. 5.65. Found: C, 43.29; H, 4.97; N, 5.75.

**Preparation of the Tripeptide Methyl Ester V According to Method B. N-Carbobenzoxy-L-alanine Pentachlorophenyl Ester (IX).** N-Carbobenzoxy-L-alanine (13 g, 0.0583 mole) was converted into the active ester IX, employing the method described previously in 80% yield. The final product was crystallized from hot ethyl acetate–ether–petroleum ether, mp 170–171°, $[\alpha]^{25}D$ −25.1° (*c* 1.38, chloroform).

*Anal.* Calcd for $C_{17}H_{12}NO_4Cl_5$: C, 43.25; H, 2.54; N, 2.98. Found: C, 43.37; H, 2.79; N, 3.25.

**N-Carbobenzoxy-L-alanyl-γ-*t*-butyl-L-glutamic Acid Methyl Ester (X).** Active ester IX (4 g, 0.0085 mole) was coupled with L-glutamic acid diester IV, and the resulting yellow oil was chromatographed on silica gel, using 350 ml of chloroform–methanol (9:1) eluate, to furnish a colorless oil in 75% yield. The oil could not be crystallized and was used without further purification in the next step.

**L-Alanyl-γ-*t*-butyl-L-glutamic Acid Methyl Ester Hydrochloride (XI).** The oily dipeptide methyl ester X (2.7 g, 0.00645 mole) was hydrogenated by the usual method and dipeptide methyl ester hydrochloride XI was isolated in 80% yield as a thick, colorless oil which could not be crystallized and was used for the next step without further purification.

**Preparation of N-Carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic Acid Methyl Ester (V).** N-Carbobenzoxy-γ-*t*-butyl-L-glutamic acid pentachlorophenyl ester (I) (1.8 g, 0.00308 mole) was coupled with dipeptide hydrochloride XI. The resulting product was crystallized and recrystallized from ether–petroleum ether in 37% yield to afford tripeptide methyl ester V, mp 114–115°, which was undepressed on admixture with a sample prepared by method A, $[\alpha]^{25}D$ −12.53 (*c* 3, chloroform).

**Preparation of the Tripeptide Pentachlorophenyl Ester VII According to Method C. N-Carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanine Hydrazide (XII).** To a solution of N-carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanine methyl ester (II, 1 g, 0.0024 mole) in methanol (10 ml), 95% hydrazine (0.2 ml) was added. The reaction mixture was kept for 16 hr at room temperature. The solvent was then evaporated *in vacuo* and the residue crystallized from

(39) H. C. Beyerman and W. M. van den Brink, *Proc. Chem. Soc.*, 266 (1963).

methanol–ether–petroleum ether to yield 0.81 g (81%) of dipeptide hydrazide XII, mp 137–139°. Chromatography on silica gel (20 g) gave on elution with chloroform–benzene (1:9, 300 ml) 0.77 g of the hydrazide, which was crystallized from methanol–ether–petroleum ether, mp 139–140°, $[\alpha]^{25}D$ −25.2° (*c* 1.55, chloroform).

*Anal.* Calcd for $C_{20}H_{30}N_4O_6$: C, 56.87; H, 7.10; N, 13.27. Found: C, 56.72; H, 7.17; N, 13.14.

**Tripeptide Methyl Ester V.** To a suspension of dipeptide hydrazide XII (0.35 g, 0.00083 mole) in ice-cold water (50 ml), concentrated hydrochloric acid (0.3 ml) and glacial acetic acid (0.75 ml) were added until a clear solution was obtained. Sodium nitrite (0.13 g) in cold water (3 ml) was rapidly added to the cold hydrazide solution until a white gummy material separated out. This was extracted with cold ethyl acetate (two 35-ml portions) and the combined ethyl acetate solution was washed with 5% aqueous sodium bicarbonate (two 10-ml portions) and water (three 20-ml portions) and dried over sodium sulfate. To the clear ethyl acetate solution of the dipeptide azide (70 ml), γ-*t*-butyl-L-glutamic acid methyl ester hydrochloride IV (0.23 g, 0.00088 mole) and triethylamine (0.13 ml, 0.00093 mole) were added. After stirring for 24 hr at room temperature, the reaction mixture was extracted twice with 20-ml portions of 1 *N* hydrochloric acid, water, 5% aqueous sodium bicarbonate, and water. Upon drying the ethyl acetate layer with sodium sulfate and evaporation *in vacuo*, a white solid was obtained which was crystallized and recrystallized from ether–petroleum ether to furnish 0.4 g (80%) of the tripeptide methyl ester V, mp 114–115°; mixture melting point with a sample prepared according to methods A or B gave no depression; $[\alpha]^{25}D$ −12.48° (*c* 3, chloroform).

**γ-*t*-Butyl-L-glutamic Acid Pentachlorophenyl Ester Hydrochloride (XIII).** N-Carbobenzoxy-γ-*t*-butyl-L-glutamic acid pentachlorophenyl ester (I) (0.585 g, 0.001 mole) was hydrogenated as described previously and the residue obtained after the usual procedure was crystallized and recrystallized from methanol–ether to afford 84% of the hydrochloride XIII, mp 138–139°, $[\alpha]^{25}D$ +36.4° (*c* 1, methanol).

*Anal.* Calcd for $C_{15}H_{17}NO_4Cl_6$: C, 36.88; H, 3.48; N, 2.87. Found: C, 36.88; H, 3.70; N, 3.09.

**Tripeptide Pentachlorophenyl Ester (VII).** To the ethyl acetate solution of the dipeptide azide (75 ml) prepared as described above from dipeptide hydrazide XII (0.350 g, 0.00083 mole), L-glutamic acid pentachlorophenyl ester hydrochloride XIII (0.405 g, 0.00083 mole) and triethylamine (0.116 ml, 0.00083 mole) were added. The mixture was stirred for 24 hr at room temperature, and then extracted twice with 20-ml portions of 1 *N* hydrochloric acid, water, 5% aqueous sodium bicarbonate, and water. The ethyl acetate layer was dried with sodium sulfate and freed from the solvent under vacuum. The residue was crystallized and recrystallized from ethyl acetate–ether–petroleum ether to afford 0.320 g (46%) of tripeptide active ester VII, mp 132–133°. Two crystallizations from methanol gave the high-melting form, mp 151–152°. Recrystallization of the methanol-crystallized product from ethyl acetate–ether–petroleum ether gave back the low-melting form, mp 132–133°. Admixture with the corresponding low- and high-melting forms prepared by method A gave no depression; $[\alpha]^{25}D$ −13.5° (*c* 2, chloroform).

**Procedure for the Polymerization of a Peptide Pentachlorophenyl Ester. Poly-γ-*t*-butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic Acid (XVII).** To a solution of tripeptide active ester hydrochloride VIII (1.80 g, 0.002 mole) in dimethyl sulfoxide (1.5 ml), triethylamine (0.58 ml, 0.004 mole) was added. The reaction mixture turned viscous in 15 min and it was kept at room temperature with shaking for 16 hr. After trituration with dry ether (150 ml), the reaction mixture was centrifuged. The solid residue was triturated and centrifuged with ether (three 50-ml portions), water (two 40-ml portions), and ether (three 50-ml portions), to afford 0.510 g (58.8%) of the *t*-butyl polymer XVII. The infrared spectrum exhibited peaks at 6.05 (amide I), 6.48 (amide II), and 5.78 μ (*t*-butyl ester); the pentachlorophenyl ester peak had completely disappeared.

*Anal.* Calcd for $(C_{21}H_{35}N_3O_7 \cdot 0.5H_2O)_n$: C, 55.05; H, 8.18; N, 9.55. Found: C, 55.00; H, 7.60; N, 9.31.

**Removal of *t*-Butyl Protecting Groups from the Polymer. Poly-L-glutamyl-L-alanyl-L-glutamic Acid (XVIII).** A solution of poly-γ-*t*-butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic acid (XVII) (0.30 g, 0.00068 mole) in 90% trifluoroacetic acid (4 ml) was kept at room temperature for 50 min. The reaction mixture was treated with ether (200 ml) and centrifuged, and the solid residue was triturated and centrifuged with ether (two 40-ml portions) to give the free polymer XVIII, 0.190 g. (85%). The infrared spectrum showed peaks at 6.04 (amide I), 6.45 (amide II), and a broad peak at 3.75–3.84 μ (COOH). The *t*-butyl ester peak had completely disappeared. The free polymer XVIII (0.180 g) was dissolved in distilled water (4 ml) and dialyzed against six 250-ml portions of distilled water for 16 hr. The polymer solution after dialysis was lyophilized to yield 0.090 g (50%) of a white solid; intrinsic viscosity 0.12 dl/g in dichloroacetic acid, measured with a Cannon–Ubbelohde viscometer (150K 200).

*Anal.* Calcd for $(C_{13}H_{19}H_3O_7 \cdot 0.5H_2O)_n$: C, 46.1; H, 5.92; N, 12.42; equiv wt, 169. Found: C, 46.05; H, 6.28; N, 12.2; equiv wt, 173.5.

The crude free polymer XVIII (0.080 g), obtained from another experiment as described above, was dissolved in distilled water (4 ml) and dialyzed for 96 hr against 250-ml portions of distilled water; the water was changed every 12 hr. The polymer solution after lyophilization afforded 0.020 g (25%) of a white fluffy solid material. The intrinsic viscosity of this sample was 0.308 dl/g in dichloroacetic acid.

**Molecular Weight Determinations. A. Number Average Molecular Weight (DNP Method).** To a solution of dialyzed poly-L-glutamyl-L-alanyl-L-glutamic acid (XVIII, 0.010 g), intrinsic viscosity 0.308 dl/g, sodium bicarbonate (0.017 g), and 0.025 *M* borax (0.5 ml) in water (1 ml), dinitrofluorobenzene (0.030 g) in ethanol (1 ml) was added. The mixture was kept at 40° for 2 hr, and then freed from the solvents under reduced pressure. The residue was dissolved in water (5 ml) and extracted with ether (three 15-ml portions) to remove unreacted dinitrofluorobenzene. The aqueous layer was acidified with 3 *N* hydrochloric acid (10 ml) and extracted with ether (three 20-ml portions) to remove dinitrophenol. The acidic solution was filtered and the total volume made up to 25 ml with 3 *N* hydrochloric acid. The absorbance of this solution was 0.357 measured at 360 mμ; a molecular weight of 16,400 was calculated from this value by comparing it with the absorbance values of known concentrations of DNP–glutamic acid in 3 *N* hydrochloric acid. Further confirmation of the number average molecular weight was achieved by determining the amount of DNP–glutamic acid obtained after hydrolysis of the DNP–polymers. A portion of the acidic solution (23 ml) of the DNP–polymers was evaporated under reduced pressure and the residue was dissolved in 1 ml of a 10% solution of 60% perchloric acid in glacial acetic acid. The reaction mixture was transferred to a test tube. One end was drawn to a capillary and the tube was kept at 110° for 4 hr in the dark. The reaction mixture was diluted with water (5 ml) and extracted with ether (three 10-ml portions). The ether solution was evaporated and the residue was diluted with ether to a volume of 5 ml. Chromatography on Whatman No. 3MM paper (*n*-butyl alcohol–acetic acid–water, 4:1:1) showed a single spot identical with that of the DNP–glutamic acid. The absorbance of this solution was 1.1 measured at 360 mμ; from this value a molecular weight of 14,400 was calculated by comparing it with the absorbance value of known concentrations of DNP–glutamic acid in ether.

For a control experiment DNP–glutamic acid (2 mg) was dissolved in ether (20 ml). The absorbance of the ether solution at 360 mμ was 2.75. The ether solution (10 ml) was evaporated and he residue was treated with sodium bicarbonate (5 mg), 0.025 *M* borax (0.16 ml), and 3 *N* hydrochloric acid (10 ml). The solution was evaporated under reduced pressure and the residue was dissolved in 1 ml. of a 10% solution of 60% perchloric acid in glacial acetic acid. The reaction mixture was transferred to a test tube, the end of it was drawn to a capillary, and the tube was kept at 110° for 4 hr in the dark. The mixture was diluted with water (5 ml) and extracted with ether (three 10-ml portions). The ether extract was evaporated and the residue was redissolved in ether to obtain a total volume of 10 ml. The absorbance of this solution at 360 mμ was 2.65, which indicated a loss of 3.5% of DNP–glutamic acid during the course of the above treatment. In a second control experiment carried out under identical conditions, the loss of DNP–glutamic acid was 5.5%.

**B. Weight Average Molecular Weight.** The weight average molecular weight was obtained from the sedimentation velocity studies carried out on a Spinco Model E centrifuge. Polymer XV, (dialyzed for 96 hr, intrinsic viscosity 0.308 dl/g) was run in 0.5 *M* tris buffer solution at pH 7.6, at a concentration of 1.1% and at a speed of 59,780 rpm. The polymer sedimented under a single peak and gave the values for sedimentation $s_{20_w}^{app} = 1$ S, and for diffusion $D_{20_w}^{app} = 6.49 \times 10^{-7}$ cm²/sec. From these values the molecular weight of approximately 25,000 was calculated using an assumed partial specific volume of 0.72.

**Determination of the Optical Purity of the Intermediate Peptides and of the Polypeptide XVIII. A. Optical Purity of N-Carbobenzoxy-γ-*t*-butyl-L-glutamic Acid Pentachlorophenyl Ester.** A solution of L-glutamic acid diester I (0.585 g, 0.001 mole) in trifluoroacetic acid (1 ml) and 6 *N* hydrochloric acid (2 ml) was refluxed for 24 hr under a carbon dioxide atmosphere. Under identical conditions a solution of L-glutamic acid (0.147 g, 0.001 mole), pentachlorophenol (0.266 g, 0.001 mole), and benzyl alcohol (0.1 ml) was refluxed with the same amounts of trifluoroacetic acid (1 ml) and 6 *N* hydrochloric acid (2 ml), to serve as a control. Both the sample and the control reaction mixtures were filtered from pentachlorophenol, which was washed with 6 *N* hydrochloric acid (3 ml), and the washings were combined with the filtrates. The filtrates were evaporated to dryness under reduced pressure and the residues dissolved in small amounts of 5 *N* hydrochloric acid; the final volume in each case was made up to 3 ml. Optical rotations were measured at 565 mμ, using a mercury (type 5H Hanovia) quartz lamp as light source: observed rotation for the sample was +1.322° and $[\alpha]^{30}_{565\ m\mu}$ +26.979°; observed rotation for the control was +1.315° which gave $[\alpha]^{30}_{565\ m\mu}$ +26.837°. The calculated optical purity is practically 100 ± 2%.

**B. Optical Purity of N-Carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanine Pentachlorophenyl Ester.** A solution of dipeptide active ester III (0.656 g, 0.001 mole) in trifluoroacetic acid (1 ml) and 6 *N* hydrochloric acid (2 ml) was refluxed for 24 hr under an atmosphere of carbon dioxide. A control experiment was carried out with optically pure N-carbobenzoxy-γ-*t*-butyl-L-glutamic acid pentachlorophenyl ester (I) (0.585 g, 0.001 mole) and L-alanine (0.089 g, 0.001 mole), using the same amounts of the acids as above. Both the sample and the control were worked up as described in the previous experiment, and the final volumes made up to 5 ml in each case with 5 *N* hydrochloric acid. Observed rotation for the sample was +0.930° which gave $[\alpha]^{30}_{565m\mu}$ +19.701° (combined rotation of the expected L-glutamic acid (0.147 g), and L-alanine (0.089 g)). Observed rotation for the control was +0.933° which gave $[\alpha]^{30}_{565\ m\mu}$ +19.77°. This would indicate 99.68 ± 2% optical purity of the sample. However, if glutamic acid did not racemize at all and only alanine racemized, then the optical purity of alanine could be calculated as follows. The ratio of the amounts (in grams) of alanine to glutamic acid after total hydrolysis is 1:1.651, and the ratio of the specific rotation values of alanine to glutamic acid, measured at 30° and 565 mμ, is 14.4:33.0. The contribution to the specific rotation of the mixture (1 mole of glutamic acid and 1 mole of alanine) would be 54.48 parts by glutamic acid and 14.4 parts by alanine. Based on this ratio (54.48:14.4), the contribution of alanine to the specific rotation of the control solution is +4.13° and that of glutamic acid is +15.64°. Thus in the sample the contribution of alanine is +4.07°. Therefore, the optical purity of alanine is 98.45 ± 2%. Calculated the same way the optical purity of glutamic acid is 99.59 ± 2%.

**C. Optical Purity of N-Carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic Acid Pentachlorophenyl Ester.** The tripeptide active ester VII (0.841 g, 0.001 mole) was completely hydrolyzed as described above. In a control experiment L-glutamic acid (0.294 g, 0.002 mole), L-alanine (0.089 g, 0.001 mole), pentachlorophenol (0.266 g, 0.001 mole), and benzyl alcohol (0.1 ml) were treated the same way. The observed rotation from the sample was +1.843° which gave $[\alpha]^{30}_{565\ m\mu}$ +24.04°, and the observed rotation for the control was +1.85° which gave $[\alpha]^{30}_{565\ m\mu}$ +24.13°. The optical purity of the tripeptide derivative, therefore, would be 99.6 ± 2%. Based on these values, the calculated minimum optical purity of glutamic acid and of alanine were 99.56 ± 2% and 96.73 ± 2%, respectively.

**D. Optical Purity of Poly-L-glutamyl-L-alanyl-L-glutamic Acid.** A solution of the free polymer XVIII (0.030 g) in 6 *N* hydrochloric acid (0.5 ml) and glacial acetic acid (0.5 ml) was refluxed for 24 hr in an atmosphere of nitrogen. The clear hydrolysate was freed from the solvents under reduced pressure. The residue was dissolved in a small amount of 5 *N* hydrochloric acid and the final volume was diluted to 1 ml with 5 *N* hydrochloric acid. A control experiment was carried out with L-glutamic acid (0.0262 g) and L-alanine (0.0079 g) using the same quantities of the acids and following the same working-up procedure as described above. The final volume of the control solution was also diluted to 1 ml with 5 *N* hydrochloric acid. The observed rotation for the sample was +0.929° which gave $[\alpha]^{30}_{565\ m\mu}$ +27.24° (calculated on the basis of the expected amounts of L-glutamic acid and L-alanine). The observed rotation for the control was +0.932° which gave $[\alpha]^{30}_{565\ m\mu}$ +27.33°, and the optical purity of the polypeptide was 99.68 ± 2%. The contribution to the specific rotations of the mixture (2 moles of glutamic acid and 1 mole of alanine) would be 7.568 parts by glutamic acid and 1 part by alanine. Based on these values, the contribution of alanine to the specific rotation of the control solution is +3.19° and that of glutamic acid is +24.14°. Thus in the sample the contribution of alanine is +3.10° and, therefore, the optical purity of alanine is 97.24 ± 2%. Calculated the same way the optical purity of glutamic acid is 99.63 ± 2%.

**Preparation and Polymerization of Tripeptide Pentachlorophenyl Ester XV. N-Carbobenzoxyglycylglycyl-L-phenylalanine Pentachlorophenyl Ester (XIV).** N-Carbobenzoxyglycylglycyl-L-phenylalanine[40] (2.0 g, 0.00485 mole) was converted into pentachlorophenyl active ester employing the usual method, and the reaction product, a white solid, was crystallized from ethyl acetate–ether–petroleum ether to afford the tripeptide active ester XIV in 78% yield, mp 112–113°, $[\alpha]^{25}D$ −10° (*c* 1, chloroform).

*Anal.* Calcd for $C_{27}H_{22}N_3O_6Cl_5$: C, 48.99; H, 3.33; N, 6.35. Found: C, 49.15; H, 3.27; N, 6.86.

**Glycylglycyl-L-phenylalanine Pentachlorophenyl Ester Hydrochloride (XV).** The tripeptide active ester XIV (1.322 g, 0.002 mole) was hydrogenated, using the conditions described previously, and the hydrochloride XV was crystallized from methanol–ether in 80% yield, mp 142–144° dec. Two recrystallizations from the same solvents raised the melting point to 144–145° dec, $[\alpha]^{28}D$ −8° (*c* 2, methanol).

*Anal.* Calcd for $C_{19}H_{17}N_3O_4Cl_6$: C, 40.48; H, 3.02; N, 7.45. Found: C, 40.61; H, 3.25; N, 7.64.

**Polyglycylglycyl-L-phenylalanine (XVI).** To a solution of the tripeptide active ester hydrochloride XV (1.128 g, 0.002 mole) in dimethyl sulfoxide (1.2 ml), triethylamine (0.50 ml, 0.004 mole) was added. The reaction mixture was shaken at room temperature for 16 hr, and then triturated with ether (150 ml). The ether suspension was centrifuged and the residue was triturated again and centrifuged with ether (three 50-ml portions), water (three 30-ml portions), and ether (three 30-ml portions) to afford 0.44 g (92%) of polyglycylglycyl-L-phenylalanine. The infrared spectrum showed peaks at 6.05 (amide I) and 6.5 μ (amide II); the pentachlorophenyl ester peak had completely disappeared. The polypeptide XVI (0.35 g) was dissolved in dimethylformamide (4 ml) and dialyzed against five 100-ml portions of dimethylformamide for 48 hr. After dialysis the polypeptide solution was concentrated under reduced pressure and the residue was triturated with ether (100 ml). The ether suspension was centrifuged and the residue was washed with ether (three 50-ml portions) to afford 0.095 g (27%) of the purified polypeptide. Intrinsic viscosity was 0.284 dl/g in dichloroacetic acid.

*Anal.* Calcd for $(C_{13}H_{13}N_3O_3 \cdot 0.5H_2O)_n$: C, 57.75; H, 5.99; N, 15.56. Found: C, 57.47; H, 6.13; N, 16.10.

**Optical Purity of Polyglycylglycyl-L-phenylalanine (XVI).** Polyglycylglycyl-L-phenylalanine (XVI) (0.054 g, 0.0002 mole) was hydrolyzed in 6 *N* hydrochloric acid (2 ml) under reflux for 48 hr. The clear solution was evaporated under reduced pressure, the residue was dissolved in a small amount of 5 *N* hydrochloric acid, and the final volume was diluted to 1 ml with the same solvent. In a control experiment L-phenylalanine (0.033 g, 0.0002 mole) and glycine (0.030 g, 0.0002 mole) were treated under the same conditions. After the evaporation of the solvent under reduced pressure, the final volume of the control solution was also diluted to 1 ml with 5 *N* hydrochloric acid. The observed rotation for the sample was −0.243° which gave $[\alpha]^{28}D$ −7.36° (calculated on the basis of the expected amount of L-phenylalanine). The observed rotation for the control was −0.246°, $[\alpha]^{28}D$ −7.45° (*c* 3.3, 5 *N* hydrochloric acid). The optical purity calculated from these values was 98.78 ± 2%.

**Preparation of γ-Tripeptide Free Acid XXXII. N-Carbobenzoxy-α-*t*-butyl-L-glutamyl-L-alanine Methyl Ester (XXVIII).** N-Carbobenzoxy-α-*t*-butyl-L-glutamic acid pentachlorophenyl ester (10 g, 0.017 mole) was coupled with L-alanine methyl ester hydrochloride and the reaction was worked up the same way as for the α-dipeptide methyl ester II. γ-Dipeptide methyl ester XXVIII was isolated in 75% yield after crystallization from ether–petroleum ether, mp 98–100°. Two more recrystallizations from the same solvents raised the melting point to 101–102°, $[\alpha]^{26}D$ +3° (*c* 3.16, chloroform).

*Anal.* Calcd for $C_{21}H_{30}N_2O_7$: C, 59.60; H, 7.10; N, 6.65. Found: C, 59.46; H, 6.87; N, 6.72.

**N-Carbobenzoxy-α-*t*-butyl-L-glutamyl-L-alanine (XXIX).** The dipeptide methyl ester XXVIII (5 g, 0.0108 mole) was saponified using the usual method, and the saponified product, a white solid,

(40) D. Ben-Ishai, *J. Org. Chem.*, **19**, 52 (1954).

was crystallized from ethyl acetate–ether in 97% yield to afford the dipeptide free acid XXIX, mp 144–145°, $[\alpha]^{25}D$ −29.5° (*c* 2.05, methanol).

*Anal.* Calcd for $C_{20}H_{28}N_2O_7$: C, 58.88; H, 6.86; N, 6.86. Found: C, 58.75; H, 6.78; N, 6.99.

**N-Carbobenzoxy-α-*t*-butyl-L-glutamyl-L-alanine Pentachlorophenyl Ester (XXX).** The dipeptide free acid XXIX (4.08 g, 0.01 mole) was converted into the pentachlorophenyl active ester as described for the preparation of the α-dipeptide pentachlorophenyl ester III. Dipeptide active ester XXX was isolated in 50% yield after crystallization from methanol, mp 132–138°. Two recrystallizations from ethyl acetate–ether–petroleum ether raised the melting point to 141–142°, $[\alpha]^{26}D$ +3.86 (*c* 0.88, chloroform).

*Anal.* Calcd for $C_{26}H_{27}N_2O_7Cl_5$: C, 47.50; H, 4.12; N, 4.27. Found: C, 47.68; H, 4.37; N, 4.24.

**N-Carbobenzoxy-α-*t*-butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic Acid Methyl Ester (XXXI).** Dipeptide active ester XXX (1.5 g, 0.00228 mole) was coupled with glutamic acid diester hydrochloride IV and the reaction mixture was worked up as described for the α-tripeptide methyl ester V, yielding 72% of the γ-tripeptide methyl ester XXXI, after crystallization from ether–petroleum ether, mp 69–72°. Two recrystallizations from the same solvents raised the melting point to 72–73°, $[\alpha]^{26}D$ −6.8° (*c* 2, chloroform).

*Anal.* Calcd for $C_{30}H_{46}N_3O_{10}$: C, 59.2; H, 7.3; N, 6.9. Found: C, 59.04; H, 7.46; N, 7.16.

**N-Carbobenzoxy-α-*t*-butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic Acid (XXXII).** Tripeptide methyl ester XXXI (0.607 g, 0.001 mole) was saponified as described previously and the saponified product, a white solid, was crystallized from ether–petroleum ether to yield 83% of the tripeptide free acid XXXII, mp 118–119°, $[\alpha]^{26}D$ +10° (*c* 0.5, chloroform).

*Anal.* Calcd for $C_{29}H_{43}N_3O_{10}$: C, 58.71; H, 7.25; N, 7.09. Found: C, 58.60; H, 7.39; N, 7.35.

**Transpeptidation Studies.** N-Carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic acid (VI, 0.030 g) was dissolved in 90% trifluoroacetic acid (1 ml) and left at room temperature for 50 min. The trifluoroacetic acid was removed under reduced pressure and the residue chromatographed on Whatman No. 3MM paper, in different solvent systems, at concentrations ranging from 30 to 4000 μg; in this case less than 1% of a tripeptide tricarboxylic acid could be detected when the chromatograms were sprayed with bromphenol blue. The solvent systems used were *n*-butyl alcohol saturated with water (descending), $R_f$ 0.85; *n*-butyl alcohol–water–acetic acid (4:1:1) (ascending), $R_f$ 0.89; *n*-butyl alcohol saturated with water and phenol–water (77:23) (two-dimensional ascending), $R_f$ 0.89 and 0.72; pyridine–water (4:1) (ascending), $R_f$ 0.79; phenyl–citric acid–disodium phosphate buffer, pH 7.6 (100:20) (ascending), $R_f$ 0.79. Similarly N-carbobenzoxy-α-*t*-butyl-γ-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic acid (XXXII) (0.030 g) was treated with 90% trifluoroacetic acid (1 ml) at room temperature for 50 min. After the removal of trifluoroacetic acid under reduced pressure, the residue was chromatographed on the same paper and in the solvent systems used for the α-tripeptide, as described above. In most of the solvent systems, though a single spot was observed, the $R_f$ values of the γ-tripeptide tricarboxylic acid were identical with those of the α-tripeptide. The most satisfactory solvent systems were pyridine–water (4:1) and phenol–citric acid–disodium phosphate buffer, pH 7.6 (100:20). The $R_f$ values with these solvent systems for the γ-tripeptide tricarboxylic acid were 0.68 and 0.62, respectively. In another experiment 3 mg each of α- and γ-tripeptides VI and XXXII were treated separately with trifluoroacetic acid (0.5 ml) for 50 min at room temperature. After the removal of trifluoroacetic acid, the residues were chromatographed separately and as a mixture. The conditions of chromatography were identical with those described above; the $R_f$ values of α- and γ-tripeptide tricarboxylic acids were identical with those described above and they separated very well into two spots. Since only one spot was observed at concentrations ranging from 30 to 4000 μg for both α- and γ-tripeptide tricarboxylic acids, it was concluded that they were 99% pure α- and γ-peptides.

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# A New Synthesis of Oxytocin Using S-Acyl Cysteines as Intermediates[1,2]

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**Abstract:** The fully S,N-protected derivatives (VI–IX) of the nonapeptide amide, L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, have been prepared by a step-by-step synthesis, using new N-protecting groups (the *t*-butyloxycarbonyl and the *o*-nitrophenylsulfenyl groups) in addition to the classical carbobenzoxy and new S-protecting groups (the S-benzoyl and S-carbobenzoxy groups). The selective removal of the S-protecting groups from compounds VI–IX by methanolysis afforded the N-protected oxytoceines (X–XII). Oxidation of the free thiol groups of X and XII by 1,2-diiodoethane led to the formation of N-carbobenzoxy- (XIII), and N-*t*-butyloxycarbonyl-oxytocin, (XIV), respectively. The peptide obtained after decarbobenzoxylation of the N-carbobenzoxy-oxytocin (XIII) was purified by countercurrent distribution and partition chromatography on Sephadex. The isolated material exhibited the chemical, physical, and biological activities of oxytocin.

Oxytocin was isolated in highly purified form by du Vigneaud, *et al.*,[3] and its structure was postulated[4] and proved by synthesis.[5] Since the first, classical synthesis of du Vigneaud, several other oxytocin syntheses were reported[6–11] from various labora-

(1) This investigation was supported by the Royal Hellenic Research Foundation, to which the author is greatly indebted.

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