Synthesis of Polypeptides with Known Repeating Sequence of Amino Acids

Sir:

Considerable attention has recently been given to the preparation of polypeptides with known repeating sequence of amino acids, especially those which contain trifunctional amino acids and approach the molecular weight of proteins.[1,2] There has been an increasing demand[1-3] for such polymers; however, no method which would satisfy the various requirements for their preparation has been developed so far.

The main difficulty in the preparation of such polypeptides[4] using the established coupling methods,[5] *e.g.*, mixed anhydrides and dicyclohexylcarbodiimide, is the undesired side reaction[5] which can lead to early termination of polymerization. On the other hand, the thiophenyl and *p*-nitrophenyl active esters[5] cannot be used for polymerization[4,6] when the N-protecting groups are removed by catalytic hydrogenation.

We wish to report now a general method for the preparation of polypeptides with known repeating sequence of amino acids using pentachlorophenyl active esters.[7] This method has the following advantages: (a) Pentachlorophenyl esters make an excellent combination with carbobenzoxy as well as with *t*-butyl protecting groups when peptides containing trifunctional amino acids are polymerized. In this combination the N-protecting carbobenzoxy group is selectively removed by catalytic hydrogenation,[8] leaving the active ester and *t*-butyl groups intact. On the other hand, the hydrogen bromide cleavage of the N-carbobenzoxy groups would rule out the use of *t*-butyl groups. The use of *t*-butyl ester protecting groups in the preparation of polypeptides containing aspartic or glutamic acids[9] is essential in order to avoid the extensive transpeptidation reactions,[10] caused by alkaline hydrolysis of an alkyl or a benzyl ester group.[11,12] (b) Pentachlorophenyl esters of amino acid and peptide derivatives are easier to purify since they are frequently higher melting compounds than other active esters.[13] High purity of the C- or N-activated peptide intermediates is essential for polymerization purposes in order to avoid undesired termination reactions.

Pentachlorophenyl active esters are among the most active esters,[7] and the polypeptides obtained by this method usually give higher molecular weights when compared with the molecular weights of polypeptides obtained by other methods[2,4] as determined by end-group analysis and viscometry.[14]

The preparation of different types of polypeptides with known repeating sequence as well as the appropriate intermediate peptides, as discussed below, will demonstrate the usefulness of this method.

Poly-(α-L-*glutamyl*-L-*alanyl*-L-*glutamic acid*), a water-soluble polypeptide, was prepared through the following sequence of reactions: N-carbobenzoxy-γ-*t*-butyl-L-glutamic acid pentachlorophenyl ester (I), m.p. 94–95°, $[\alpha]^{27}D$ −12° (*c* 3.38, chloroform), was coupled with L-alanine methyl ester hydrochloride to yield 69% N-carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanine methyl ester (II), m.p. 97–98°, $[\alpha]^{27}D$ −6.7° (*c* 3.24, chloroform). The saponified product of dipeptide II gave with pentachlorophenol and dicyclohexylcarbodiimide (DCCI) N-carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanine pentachlorophenyl ester (III) in 64% yield, m.p. 170–171°, $[\alpha]^{25}D$ −18.5° (*c* 1.33, chloroform). The coupling of III with γ-*t*-butyl-L-glutamic acid methyl ester hydrochloride afforded N-carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic acid methyl ester (IV) in 79% yield, m.p. 112–113°, $[\alpha]^{25}D$ −12.5° (*c* 1.6, chloroform). The saponified product of tripeptide IV (m.p. 58–59°, 80% yield) was converted in 75% yield to N-carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic acid pentachlorophenyl ester (V), m.p. 151–152°, $[\alpha]^{27}D$ −13.2° (*c* 2.0, chloroform). Analytical values for the above intermediates are in agreement with the calculated ones.

Tripeptide active ester V was hydrogenated in the presence of palladium on charcoal catalyst and 1 mole

(1) E. R. Blout, "Polyamino Acids, Polypeptides, and Proteins," M. A. Stahmann, Ed., The University of Wisconsin Press, Madison, Wis., 1962, pp. 3–11.

(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.

(3) P. H. Maurer, *Progr. Allergy*, **8**, 1 (1964).

(4) (a) J. Kovacs, ref. 1, pp. 37–47; (b) V. Bruckner and J. Kovacs, *Acta Chim. Acad. Sci. Hung.*, **12**, 363 (1957); (c) V. Bruckner, M. Kajtar, J. Kovacs, H. Nagy, and J. Wein, *Tetrahedron*, **2**, 211 (1958).

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

(6) J. Kovacs, R. Ballina, and R. Rodin, *Chem. Ind.* (London), 1955 (1963); D. F. DeTar, *et al.*, *J. Am. Chem. Soc.*, **85**, 2873 (1963).

(7) Little attention has been given to the use of pentachlorophenyl active esters, though G. Kupryszevski (*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. Lehmann (*Helv. Chim. Acta*, **46**, 1887 (1963) and J. Pless and R. H. Boissonnas (*ibid.*, **46**, 1609, (1963)) studied the kinetics of the aminolysis of Z–Phe 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.

(8) When the hydrogenation of the N-protected peptide pentachlorophenyl esters, suspended in methanol–5% acetic acid, is carried out with activated 10% palladium on charcoal catalyst, in addition to the removal of carbobenzoxy group chlorine is also removed from the pentachlorophenyl residue as hydrogen chloride (R. Ballina's observation). Systematic investigation of this reaction (by R. A. Giannotti) on various pentachlorophenyl esters indicated that when the hydrogenation was carried out in the presence of 1 mole of hydrogen chloride in methanol, no chlorine was removed from the benzene ring (5–15 min. reaction time for 2–3 g. of material with 200–300 mg. of catalyst.)

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

(10) J. Kovacs, K. Medzihradszky, and V. Bruckner, *Naturwiss.*, **41**, 450 (1954); J. Kovacs, I. Konyves, and J. Csaszar, *ibid.*, **41**, 575 (1954).

(11) E. Sondheimer and R. W. Holley, *J. Am. Chem. Soc.*, **76**, 2567 (1954).

(12) Transpeptidation reaction was studied on glutamyl peptide IV. After treatment of IV with trifluoroacetic acid, 50 to 300 μg. of the crude reaction product was chromatographed in two solvent systems which seems to indicate that only one component was present.

(13) In our experience the advantage of the pentachlorophenyl ester is evident when the other active esters have low melting points; *e.g.*, Z-Glu-α-*t*-butyl-γ-*p*-nitrophenyl ester, m.p. 49–50°, and its 2,4,5-trichlorophenyl ester is an oil while the pentachlorophenyl ester melts at 122°.

(14) Average molecular weights determined by the DNP method indicate that Doty's calibration curve for poly-γ-benzyl-L-glutamate (Doty, *et al.*, *J. Am. Chem. Soc.*, **78**, 947 (1956)) is useful at least for a rough estimation of the molecular weights of these polymers.

of hydrogen chloride, and the resulting tripeptide active ester hydrochloride was polymerized in dimethylformamide (50% solution) in the presence of triethylamine to poly-(γ-*t*-butyl-L-glutamyl-L-alanyl-γ-*t*-butyl-L-glutamic acid) (VI) in 42% yield. The *t*-butyl ester groups from polymer VI were removed by 90% trifluoracetic acid and the water-soluble poly-α-L-glutamyl-L-alanyl-L-glutamic acid (VII) was obtained in 94% yield. Further purification was achieved by dialysis[15] for the removal of low molecular weight polypeptides and possible cyclic peptides. *Anal.* Calcd. for $(C_{13}H_{19}N_3O_7.0.5H_2O)_\infty$: C, 46.1; H, 5.94; N, 12.4; equiv. wt., 169. Found: C, 46.34; H, 6.41; N, 12.10; equiv. wt., 173.5; intrinsic viscosity in dichloroacetic acid, 0.12 dl./g. Molecular weight as determined by DNP method was 20,000. Retention of optical purity was 98 ± 2%.[16]

Similarly N-carbobenzoxyglycylglycyl-L-phenylalanine pentachlorophenyl ester was polymerized to poly-(glycylglycyl-L-phenylalanine) in 58% yield.

Polypeptides with known repeating sequence of amino acids containing other than α-peptide bonds, *e.g.*, γ-glutamyl residues, are also important.[17] Preparation of poly-(γ-L-glutamyl-γ-aminobutyric acid) (VIII) from N-carbobenzoxy-α-*t*-butyl-L-glutamyl-γ-aminobutyric acid pentachlorophenyl ester (IX), m.p. 175–176°, $[\alpha]^{26}D$ −3° (*c* 2.0, chloroform), was achieved in 49% over-all yield after dialysis; intrinsic viscosity 0.135 dl./g. *Anal.* Calcd. for $(C_9H_{14}N_2O_4)_\infty$: C, 50.51; H, 6.55; N, 13.10. Found: C, 51.03; H, 7.08; N, 12.6.

Other polypeptides containing different amino acid residues were also prepared by this general procedure.

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(15) During the extensive dialysis the loss for various batches was between 38 and 62%.

(16) The retention of the optical purity was established by determining the specific rotation of the total hydrolysate of the polypeptides and comparing that with the specific rotation of the corresponding amino acids treated under the same conditions.

(17) J. Kovacs, U. R. Ghatak, G. N. Schmit, F. Koide, J. W. Goodman, and D. E. Nitecki, International Symposium on the Chemistry of Natural Products, Kyoto, Japan, April 1964; Abstracts of Papers, pp. 175–178.

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Poly-β-L-aspartic Acid. Synthesis through Pentachlorophenyl Active Ester and Conformational Studies

Sir:

Utilization of the pentachlorophenyl active ester method for the synthesis of poly-β-L-aspartic acid has resulted in optically pure high molecular weight polypeptide, which was needed for biological and physical chemical investigations. The importance of optically pure poly-β-aspartic acids for immunochemical studies was emphasized in our earlier paper,[1] where a synthesis through *p*-nitrophenyl ester was also reported. Search for better polymerization methods led to the use of trichlorophenyl and later of pentachlorophenyl active esters.[2]

N-Carbobenzoxy-α-benzyl-L-aspartic acid pentachlorophenyl ester (I), m.p. 151–152°, $[\alpha]^{26}D$ 20.0° (*c* 2.36, chloroform), was obtained in 95% yield from N-carbobenzoxy-α-benzyl-L-aspartate and pentachlorophenol using the DCCI method.[3] *Anal.* Calcd. for $C_{25}H_{18}O_6NCl_5$: C, 49.57; H, 3.00; N, 2.31. Found: C, 49.57; H, 3.24; N, 2.35. Hydrogen bromide cleavage of I [10 g. of I in 140 ml. of 8.5% solution of hydrogen bromide in acetic acid–dichloroacetic acid (1:6)] gave after two recrystallizations from absolute alcohol 84% α-benzyl-β-pentachlorophenyl-L-aspartate hydrobromide (II), m.p. 201–202°, $[\alpha]^{26}D$ 7.12° (*c* 3.38, dimethylformamide). *Anal.* Calcd. for $C_{17}H_{13}O_4NCl_5Br$: C, 36.96; H, 2.37; N, 2.54. Found: C, 37.04; H, 2.46; N, 2.87. Active ester II polymerized readily in dimethylformamide in the presence of a tertiary amine to poly-β-(α-benzyl)-L-aspartic acid (III) in 95% yield of which 62% was a polypeptide with an average molecular weight of 10,000, as determined by end-group analysis.[4] (During the polymerization of II, the higher molecular weight polypeptide precipitated from the dimethylformamide solution (62%) and the lower molecular weight fraction was precipitated by ether.) The intrinsic viscosity of III in dichloroacetic acid is 0.09 dl./g. *Anal.* Calcd. for $(C_{11}H_{11}O_3N)_\infty$: C, 64.38; H, 5.40; N, 6.88. Found: C, 63.90; H, 5.41; N, 6.76. Catalytic hydrogenation of III gave optically pure water-soluble poly-β-L-aspartic acid (IV) in 99% yield. After dialysis polypeptide IV was obtained in 50% yield; intrinsic viscosity in dichloroacetic acid 0.11 dl./g.; molecular weight, as determined by the DNP method, 19,000; equivalent weight 125.1, calcd. 124. *Anal.* Calcd. for $(C_4H_5O_3N.0.5\ H_2O)_\infty$; C, 38.79; H, 4.80; N, 11.21. Found: C, 39.01; H, 4.55; N, 11.42.

The optical purity of the dialyzed sample was established by determining the optical purity of the aspartic acid after total hydrolysis; 58.6 mg. of sample was refluxed for 24 hr. in 3 ml. of 12 *N* hydrochloric acid, the solution was evaporated to dryness, the residue was dried for 24 hr. over sodium hydroxide and phosphorus pentoxide and then dissolved in 5 ml. of water, and the specific rotation was determined with a Rudolph Model 200AS-80Q spectropolarimeter; $[\alpha]^{26}D$ 19.65° (*c* 1.25, water). As a control, 68.3 mg. of aspartic acid was treated in the same manner; $[\alpha]^{26}D$ 20.03° (*c* 1.35, water), which indicated at least 98.1% optical purity. The exclusive presence of β-peptide linkages was determined by Hofmann degradation.[5]

Poly-β-L-aspartic acid was also prepared through the α-*t*-butyl ester derivative. N-Carbobenzoxy-α-*t*-butyl-L-aspartic acid pentachlorophenyl ester (V), m.p. 121–123°, $[\alpha]^{26}D$ 37.5° (*c* 2, chloroform) [*Anal.* Calcd. for $C_{22}H_{20}O_6NCl_5$: C, 46.20; H, 3.54; N, 2.45. Found:

(1) J. Kovacs, R. Ballina, and R. Rodin, *Chem. Ind.* (London), 1955 (1963).

(2) A few trichlorophenyl and pentachlorophenyl esters were reported first by G. Kupryszewski and M. Kaczmarek, *Roczniki Chem.*, **35**, 935, 1533 (1961); K. Stich and H. A. Lehmann [*Helv. Chim. Acta*, **46**, 1887 (1963)], studied the kinetics of aminolysis of different chlorophenyl esters.

(3) D. F. Elliot and D. W. Russell, *Biochem. J.*, **66**, 49P (1957).

(4) M. Sela and A. Berger, *J. Am. Chem. Soc.*, 77, 1893 (1955).

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