Synthesis and Conformation of Sequential Polypeptides containing ε-Benzyloxycarbonyl-lysine and Benzyl Esters of Aspartic and Glutamic Acids †

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Sequential polypeptides with the repeating units N^{ϵ}-Z-L-Lys-γ-Bzl-L-Glu and N^{ϵ}-Z-L-Lys-β-Bzl-L-Asp have been synthesized by solution polymerization of their respective succinimido-esters. Both polypeptides are helical in solution in hexafluoropropan-2-ol and in trifluoroethanol, but random in hexafluoroacetone sesquihydrate, as shown by circular dichroism. Polarized i.r. spectra of oriented films of the polypeptides are also characteristic of the α-helical conformation.

THE way in which the amino-acid sequence determines protein conformation is of great interest. Pioneer work with synthetic homopolypeptides has given much information on the possible role of individual residues in the self-organization of protein structure. Subsequent extensions to include both block and random copolymers has permitted a partial evaluation of the effect of interacting neighbours.[1] More recent work with polypeptides having ordered residue sequences has allowed the study, under controlled conditions, of the role of the interaction between neighbouring residues on the conformation of the peptide chain.[2] Since improvements in the synthetic procedures have been realized,[3] mainly in the use of active esters as polymerizing units, the syntheses of a great number of high molecular weight sequential polypeptides have been reported.

It is well established that, in helicogenic solvents as well as in the solid state, poly-(γ-benzyl L-glutamate) (PBLG) [4] and poly-(N^{ϵ}-benzyloxycarbonyl-L-lysine) (PCLL) [5] adopt a stable, right-handed α-helical conformation. Sequential polypeptides of ε-benzyloxycarbonyl-L-lysine and γ-benzyl L-glutamate also prefer that conformation in the solid state, as shown by i.r. spectroscopy.[6] On the other hand, poly-(β-benzyl L-aspartate) (PBLA) is known to form preferentially left-handed α-helices under normal conditions.[1,7] Previous studies with random copolymers of benzyl L-aspartate and benzyl L-glutamate have shown that the right-hand screw sense of the helix is retained until more than 90% benzyl

† Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature. Other abbreviations used are: DMF, *NN*-dimethylformamide; THF, tetrahydrofuran; DCC, dicyclohexylcarbodi-imide; DCHU, dicyclohexylurea.

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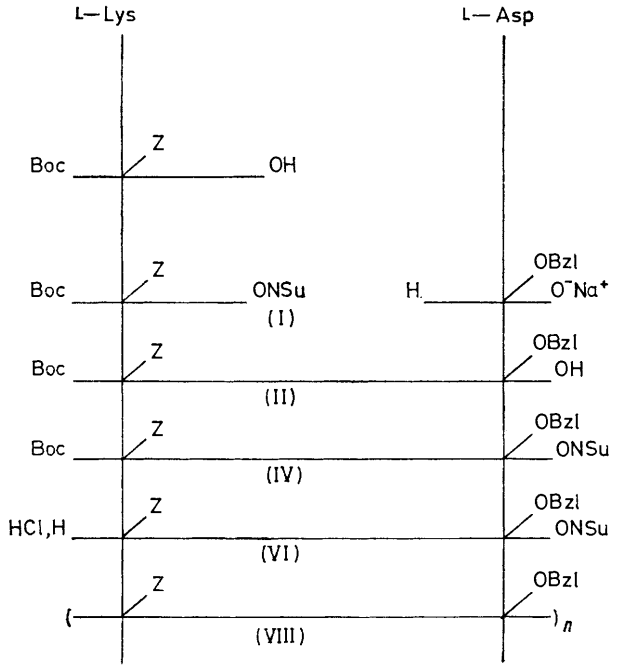
[2] B. J. Johnson, *J. Pharm. Sci.*, 1974, **63** (3), 10.
[3] H. J. Goren, *CRC Crit. Rev. Biochem.*, 1974, 197.
[4] T. Miyazawa and E. R. Blout, *J. Amer. Chem. Soc.*, 1961, **83**, 712.
[5] G. D. Fasman, M. Idelson, and E. F. Blout, *J. Amer. Chem. Soc.*, 1961, **83**. 709.
[6] R. Ledger and F. H. C. Stewart, *Austral. J. Chem.*, 1967, **20**, 2509.
[7] E. M. Bradbury, B. G. Carpenter, and R. M. Stephens, *Macromolecules*, 1972, **5**, 8.

L-aspartate was incorporated,[8] showing the greater stability of the glutamate helix. The benzyl L-aspartate and benzyl L-glutamate sequential copolypeptide also adopts the right-handed helical conformation in the solid state.[6]

We report here the synthesis of poly-N$^{\epsilon}$-Z-L-Lys-β-Bzl-L-Asp (poly-LA) and poly-N$^{\epsilon}$-Z-L-Lys-γ-Bzl-L-Glu (poly-LG), and their preliminary conformational analysis by c.d. and polarized i.r. spectroscopy.

RESULTS AND DISCUSSION

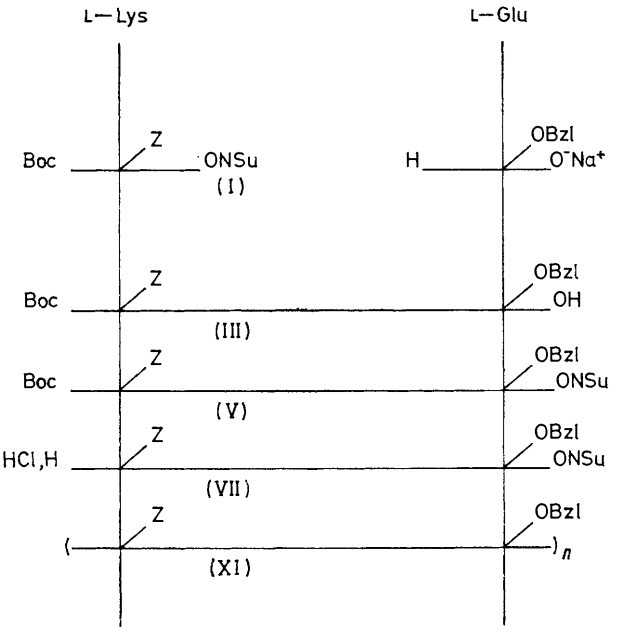
The reactions leading to poly-LG and poly-LA are summarized in Schemes 1 and 2. High yields were achieved at every step and all the intermediates were obtained in analytically pure condition, merely by washing in ethyl acetate or crystallization. The coupling reactions were performed in water–dioxan by the active succinimido-ester coupling procedure, with the *N*-protected derivative of L-lysine and the sodium salt of the aspartyl or glutamyl derivative. The carboxy-ends of the dipeptide units were also activated by formation of the succinimido-ester. We chose this particular active ester because of its successful use for the synthesis of other



SCHEME 1

sequential polypeptides,[9,10] giving good yields of high molecular weight material, with a minimum of racemization.

Prior to polymerization, the *N*-protecting Boc group of the lysine residue was removed quantitatively, by using



SCHEME 2

2N-hydrochloric acid in dioxan. Special care was taken at this crucial step to avoid non-specific hydrolysis of the acid-labile benzyloxycarbonyl and benzyl ester-protecting groups. Previous tests showed us that the deprotection is specific under these conditions.

However, the polymerization of the dimer units led to relatively low yields of polypeptides, owing to significant formation of piperazinediones. Fortunately, these side-products are easily separated from the high molecular weight material by washing with hot methanol, as shown by i.r. spectroscopy. The average degree of polymerization (*ca.* 50 residues per chain) of the polypeptides is comparable with those of other polypeptides synthesized by similar methods.[11,12]

Figure 1 shows the c.d. spectra of poly-LA in hexafluoropropan-2-ol (HFIP), trifluoroethanol (TFE), and hexafluoroacetone sesquihydrate (HFA). In HFIP and TFE, the spectra are similar to those reported for the α-helical conformation, with troughs at 222 and 208 nm and an intense positive peak at 192 nm.[13] The same can be said for the spectra of poly-LG in those two solvents (Figure 2). However, in HFA, both polypeptides display completely different spectra, that could be attributed to the random conformation.

Figures 3 and 4 show the amide A, I, and II and the ester C=O i.r. bonds exhibited by oriented films of the polypeptides oriented parallel or perpendicular to the polarized radiation. The positions and the dichroism of

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[9] A. Yaron, N. Tal, and A. Berger, *Biopolymers*, 1972, **11**, 2461.
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[12] P. M. Hardy, H. N. Rydon, and R. C. Thompson, *J.C.S. Perkin I*, 1972, **1**, 5.
[13] S. Beychok in ref. 1, p. 297.

the amide I and II bands (*ca.* 1 650 and 1 540 cm^{-1}, respectively) are characteristic [14,15] of the α-helical conformation. Moreover, the amide I band displays a

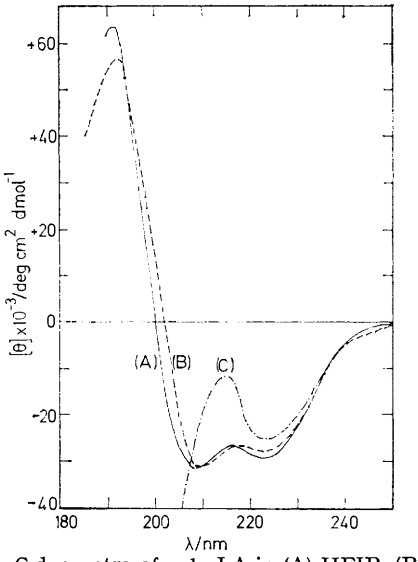

FIGURE 1 C.d. spectra of poly-LA in (A) HFIP, (B) TFE, and (C) HFA

parallel dichroism and the amide II band a perpendicular dichroism, indicating that the carbonyl chromophores of the backbone are parallel to the direction of the stretching. The same can be said about the N–H stretching band around 3 280 cm^{-1} that shows a parallel dichroism.

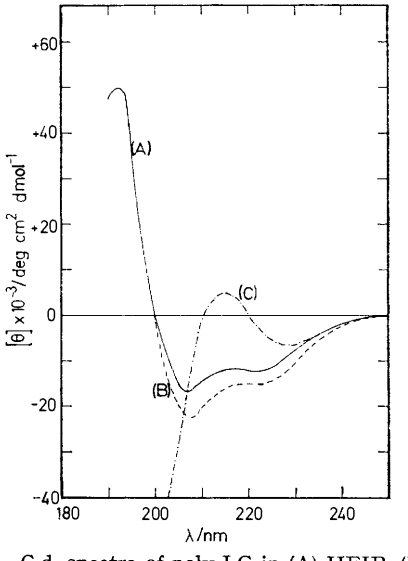

FIGURE 2 C.d. spectra of poly-LG in (A) HFIP, (B) TFE, and (C) HFA

Also, both ester bands (*ca.* 1 720 cm^{-1}) display parallel dichroism, indicating that the orientation of the side chains is influenced by the conformation of the backbone. Similar results have been reported for poly-(γ-benzyl glutamate) [16] and for poly-LG[6].

Those combined results lead us to conclude that poly-LG and poly-LA adopt preferentially the right-handed α-helical conformation in solution in ' helicogenic solvents ' like HFIP or TFE and in the solid state. However, from c.d. studies in HFA, and from preliminary helix–coil transition studies,[17] it seems that the helices formed by sequential poly-LG and poly-LA are not very

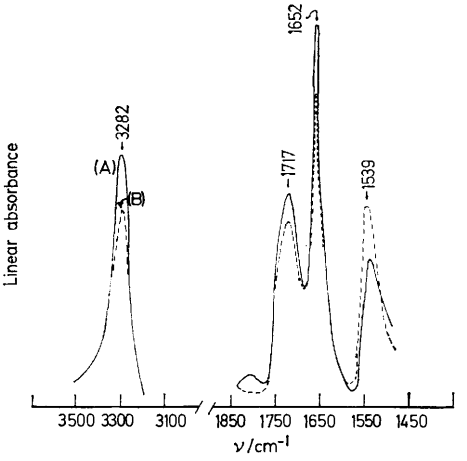

FIGURE 3 Polarized i.r. spectrum of poly-LA films from HFIP: (A) parallel orientation; (B) perpendicular orientation

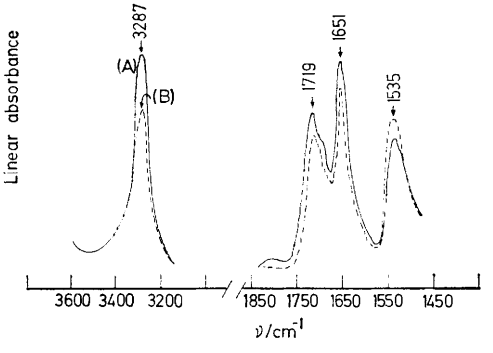

FIGURE 4 Polarized i.r. spectrum of poly-LG films from HFIP: (A) parallel orientation; (B) perpendicular orientation

stable, relative to those formed by the corresponding homopolypeptides PBLG and PCLL. Figures 1 and 2 show that the helical structure found for the two polypeptides in HFIP or TFE is disorganized when the same polypeptides are dissolved in a water-containing solvent like HFA. This destabilization of the helix in HFA can be explained by an increase of the dielectric constant of the solvent relative to HFIP or TFE, or by partial hydration of the backbone causing competition for the

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hydrogen bonds necessary to stabilize the helical structure, or by a combination of these two factors. Water has, in the case of weak helices, a denaturing effect similar to that of strong acids.[17] The relatively low molecular weight of the polypeptides is a further reason for helix instability.

Our results also show that the regular insertion of a β-benzyl-aspartyl residue as every second unit in a chain of poly-LA is not sufficient to reverse the chirality of the α-helix, as demonstrated previously with random copolypeptides of the same composition.[8]

Finally, the parallel i.r. dichroism of the ester carbonyl band at *ca.* 1 775 cm^{-1} shows that the rigid helical conformation of the polypeptides causes backbone orientation, to a certain extent, of the relatively short side-chains of benzyl aspartate and glutamate. The degree of orientation appears similar for both polypeptides from the respective dichroic ratios. Further studies will be needed in order to establish the nature of the interactions causing this orientation.

EXPERIMENTAL

N^{α}-Boc-N^{ϵ}-Z-Lys, β-Bzl-L-Asp, and γ-Bzl-L-Glu (Schwarz-Mann) were used without further purification. DCC (Aldrich) was purified by dissolving in anhydrous diethyl ether and removing insoluble material by filtration; the ether was evaporated off *in vacuo*. *N*-Hydroxysuccinimide (Aldrich) was used without further purification. Dioxan (Mallinckrodt) was purified from water and peroxides as described.[18] After distillation over sodium, it was stored under nitrogen in brown bottles, over calcium hydride, at 4 °C. 4N-Hydrochloric acid in dioxan was prepared according to the literature method and stored in sealed 10 ml ampoules at 4° C. DMF was purified as described previously.[18] The forerun was discarded and the middle fraction, b.p. 40 °C at 9 mmHg, was used. Triethylamine (Eastman) was refluxed over sodium for 2 h and fractionally distilled at atmospheric pressure (b.p. 88 °C). All the other solvents and reagents mentioned were of analytical grade and used without further purification.

M.p.s were determined with a Büchi capillary apparatus. Optical rotations were measured with a Perkin-Elmer 141 polarimeter at 589 nm (1 dm pathlength cell; 1 ml volume). I.r. spectra of intermediates were recorded for KBr pellets, with a Perkin-Elmer 621 spectrophotometer. ^{1}H N.m.r. spectra were taken with a Varian T-60 spectrometer for solutions in deuteriochloroform (tetramethylsilane as internal reference). Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory Inc., Woodside, New York. T.l.c. was carried out on precoated silica gel plates (Kodak 246 F). The solvent systems were butan-1-ol–acetic acid–water [4 : 1 : 5 (BAW5) or 4 : 1 : 1 (BAW1)], propanol–ammonium hydroxide (84 : 37) (PA), chloroform–acetone (5 : 1) (CA), acetone–acetic acid (95 : 5) (AA), and chloroform–methanol–acetone (85 : 10 : 5) (CMA). Peptide spots were located by spraying with 0.1% ninhydrin in acetone and heating (free amine) or treating the plates with hydrogen chloride vapours for 15 min, spraying with ninhydrin, and heating (Boc-amines).

The molecular weights of compounds (II) and (III) were determined by carboxy-end-group titration[19] in methanol with standardized 0.100N-sodium methoxide in methanol [Thymol Blue (0.1% in DMF) as indicator].

N^{α}-Boc-N^{ϵ}-Z-L-Lys-ONSu (I).—A mixture of N^{α}-Boc-N^{ϵ}-Z-L-Lys (4.28 g, 0.11 mol) and *N*-hydroxysuccinimide (1.30 g, 0.011 mol) was dissolved in ethyl acetate–dioxan (60 : 40; 100 ml). The solution was cooled to 0 °C and DCC (2.56 g, 0.012 mol) in ethyl acetate (15 ml) was added. After 2 h at 0 °C and 48 h at 4 °C, glacial acetic acid (3 drops) was added; after a further 2 h at 4 °C DCHU was filtered off and the solution was evaporated to an oil, which was dissolved in ethyl acetate (50 ml). The solution was set aside overnight at 4 °C, more DCHU was filtered off, another 50 ml of ethyl acetate was added, and the solution was extracted with 0.5M-sodium hydrogen carbonate (3 × 20 ml) at 0 °C and with water and evaporated. The oil was twice redissolved in diethyl ether and the solution evaporated under high vacuum. The white residual solid was pure *active ester* (I), as shown by analysis; yield 4.5 g (85%), m.p. 100—101 °C, $[\alpha]_D^{25}$ −11.7: (*c* 1.5 in $CHCl_3$); R_F^{AA} 0.83, R_F^{BAW5} 0.65; $\bar{\nu}$(KBr) 1 807, 1 780, and 1 685 cm^{-1}; τ (CD-Cl_3) 2.70 (5 H, s, Ph), 4.59 (1 H, NH), 4.71 (1 H, NH), 4.92 (2 H, s, PhCH_2), 5.45 (1 H, s, α-CH), 6.56 (2 H, d, ε-CH_2), 7.04 (4 H, s, CH_2 of succimido), and 8.55 (15 H, m, β-, γ-, δ-CH_2 and Boc CH_3s) (Found: C, 57.7; H, 6.5; N, 8.75. $C_{23}H_{31}N_3O_8$ requires C, 57.85; H, 6.05; N, 8.8%).

N^{α}-Boc-N^{ϵ}-Z-L-Lysyl-β-Bzl-L-Asp (II).—β-Bzl-L-Asp (0.7 g, 3 mmol) was dissolved in hot water (25 ml); the solution was cooled to room temperature and solid sodium hydrogen carbonate (0.27 g, 3 mmol) was added. The aqueous solution was poured into a solution of compound (I) (1.5 g, 3 mmol) in dioxan (25 ml), and a solution of sodium hydrogen carbonate (0.27 g) in water (20 ml) was added. More dioxan was added to redissolve the precipitated material. After 48 h at 4 °C, the solution was evaporated and the oil dissolved in water (50 ml); this solution was cooled to 0 °C and acidified to pH 3 with 0.5N-hydrochloric acid. Heavy precipitation occurred and the suspension was extracted with ethyl acetate (3 % 25 ml). The organic phase was washed, at 0 °C, with 0.05N-hydrochloric acid (3 %, 25 ml) and water, dried ($MgSO_4$), and evaporated. The oil was twice dissolved in diethyl ether and the solution evaporated. The semi-solid residue was dried under high vacuum and contained pure *dipeptide* (II) as shown by t.l.c. and titration; yield 1.40 g (80%); R_F^{PA} 0.76, R_F^{AA} 0.75, R_F^{BAW5} 0.76; *M* 583 (calc. 584.1).

N^{α}-Boc-N^{ϵ}-Z-L-Lysyl-γ-Bzl-L-Glu (III).—The procedure was the same as for compound (II). Compound (I) (1.5 g, 0.3 mmol), γ-Bzl-L-Glu (0.74 g, 0.3 mmol), and sodium hydrogen carbonate (0.53 g, 0.6 mmol) of $NaHCO_3$ were used. The white semi-solid obtained after drying under high vacuum contained pure *dipeptide* (III), as shown by t.l.c. and molecular weight determination; yield 1.47 g (82%); R_F^{PA} 0.77, R_F^{AA} 0.79, R_F^{BAW5} 0.76; *M* 600 (calc. 598.1).

N^{α}-Boc-N^{ϵ}-Z-L-Lys-β-Bzl-L-Asp-ONSu (IV).—Compound (II) (1.30 g, 2.2 mmol) was dissolved in dry ethyl acetate (25 ml), *N*-hydroxysuccinimide (0.26 g, 2.2 mmol) was dissolved in dry dioxan (10 ml), and the solutions were combined. The mixture was cooled to 0 °C and a solution of DCC (0.51 g, 2.4 mmol) in ethyl acetate (5 ml) was added. After 2 h at 0 °C and 72 h at 4 °C, glacial acetic acid (2 drops) was

[18] J. M. Stewart and J. D. Young in 'Solid Phase Peptide Synthesis,' Freeman, San Francisco, 1969.
[19] M. Sela and A. Berger, *J. Amer. Chem. Soc.*, 1955, **77**, 1893.

added, and after 2 h DCHU was filtered off and the solution evaporated *in vacuo*. The syrupy residue was dissolved in ethyl acetate (20 ml) and set aside overnight at 4 °C. More DCHU was filtered off, ethyl acetate (30 ml) was added, and the solution was washed, at 0 °C, with 0.5M-sodium hydrogen carbonate (3 × 30 ml) and water, and dried ($MgSO_4$). The ethyl acetate was evaporated off *in vacuo*, and ether was added and evaporated off until formation of a white solid. The powder was recrystallized (acetone–ether) to give the *product* (IV) (1.0 g, 66%), m.p. 87 °C; $[\alpha]_D^{25}$ −21.4° (*c* 2 in Me_2CO); R_F^{AA} 0.82, R_F^{CMA} 0.89 (Found: C, 59.75; N, 8.3; H, 6.25. $C_{34}H_{42}N_4O_{11}$ requires C, 59.8; N, 8.2; H, 6.15%).

N$^{\alpha}$-*Boc*-*N*$^{\epsilon}$-*Z*-L-*Lys*-γ-*Bzl*-L-*Glu*-*ONSu* (V).—The procedure was the same as for compound (IV). Compound (III) (1.46 g, 2.5 mmol), *N*-hydroxysuccinimide (0.28 g, 2.5 mmol) and DCC (0.558 g, 2.6 mmol) were used. The white solid *product* (1.2 g, 70%) contained pure (V) as shown by t.l.c. and analysis; $[\alpha]_D^{25}$ −21.9° (*c* 2 in Me_2CO acetone); R_F^{AA} 0.84, R_F^{CMA} 0.86 (Found: C, 60.2; N, 8.15; N, 6.35. $C_{35}H_{44}N_4O_{11}$ requires C, 60.35; N, 8.05; H, 6.3%).

N$^{\epsilon}$-*Z*-L-*Lys*-β-*Bzl*-L-*Asp*-*ONSu*,HCl (VI).— Compound (IV) was dissolved in 2N-hydrochloric acid in dioxan (20 ml). After 1 h at room temperature, the solution was evaporated to an oil under vacuum with exclusion of moisture. This oil was redissolved twice in dry dioxan and the solvent evaporated off. The semi-solid was suspended in dry diethyl ether and triturated to give a white powder. The suspension was centrifuged and the ether decanted. The solid was washed three times with ethyl acetate–ether and centrifuged. After decantation of the liquid, the hydrochloride was dried overnight (P_2O_5). The hygroscopic white powder was pure by t.l.c.; yield 98%; m.p. 86—88 °C; $[\alpha]_D^{25}$ −13.8° (*c* 2 in DMF); R_F^{AA} 0.06, R_F^{BAW5} 0.73, R_F^{BAW1} 0.64.

N$^{\epsilon}$-*Z*-L-*Lys*-γ-*Bzl*-*Glu*-*ONSu*,HCl (VII).—The procedure was the same as for compound (VI); compound (V) (1.1 g, 1.6 mmol) afforded the pure product (VII) as a white *solid* (1 g, quantitative), m.p. 69—71 °C; $[\alpha]_D^{25}$ 4.5° (*c* 2 in DMF); R_F^{AA} 0.06, R_F^{BAW5} 0.75, R_F^{BAW1} 0.71.

Poly-(*N*$^{\epsilon}$-*Z*-L-*Lys*-β-*Bzl*-L-*Asp*) (VIII).—Compound (VI) (0.9 g, 1.5 mmol) was dissolved in purified DMF (3 ml); the solution was cooled to 0 °C and triethylamine (0.15 g, 1.5 mmol) in DMF (1 ml) was added. The mixture solidified immediately and more DMF (3 ml) was added. The thick slurry was kept with vigorous stirring at 4 °C for 2 days. After further dilution with DMF (2 ml), the viscous suspension was stirred at room temperature for 7 days, 2 ml of DMF being added every day. The mixture was then evaporated to a paste (rotary evaporator), water was added, and the solid was centrifuged. The water was decanted and the washing with water was repeated many times. The solid was finally filtered off, washed with hot methanol and then with ether, and dried at 50 °C under vacuum for 48 h to yield the *polypeptide* (VIII) (250 mg, 36%); amino acid analysis: Lys, 1.00; Asp, 0.98; $\bar{\nu}$(KBr) 3 282, 1 720, 1 652, and 1 542 cm^{-1} (Found: C, 64.85; N, 9.1; H, 6.2. $C_{25}H_{29}N_3O_6$ requires C, 64.25; N, 9.0; H, 6.2%).

Poly-(*N*$^{\epsilon}$-*Z*-L-*Lys*-γ-*Bzl*-L-*Glu*) (IX).—The same procedure with compound (VII) (0.9 g, 1.4 mmol) and triethylamine (0.14 g, 1.4 mmol) gave the *polypeptide* (IX) (265 mg, 39%); amino-acid analysis: Lys, 1.00; Glu, 1.01; $\bar{\nu}$(KBr) 3 287, 1 719, 1 650, and 1 535 cm^{-1} (Found: C, 64.9; N, 8.9; H, 6.3. $C_{26}H_{31}N_3O_6$ requires C, 64.85; N, 8.75; H, 6.5%).

Characterization and Conformational Analysis of the Polypeptides.—*Molecular weight determination.* The molecular weights were estimated from the intrinsic viscosity–molecular weight calibration curve of Doty *et al.*[20] Intrinsic viscosities were determined in dichloroacetic acid at 25 °C with a Hewlett-Packard automatic viscometer equipped with a Cannon semi-micro Ubbelohde-type viscometer.

Amino-acid analysis. The amino-acid compositions were evaluated with a Beckman 120 automatic analyser, by use of the procedure of Spackman *et al.*,[21] after hydrolysis in 6N-hydrochloric acid, under vacuum at 110 °C for 24 h.

Circular dichroism measurements. C.d. measurements were made with a Cary 61 spectropolarimeter [path lengths 0.01, 0.1, and 1.0 cm (Hellma)]. During the measurements, the instrument was purged with nitrogen, at 40 ft^3 min^{-1}. The spectropolarimeter was calibrated with (+)-camphor-10-sulphonic acid.[22] The mean residue ellipticity, $[\theta]_\lambda$ (in deg cm^2 $dmol^{-1}$), was calculated according to the relationship $[\theta]_\lambda = \theta_\lambda(MRW)/10lc$, where θ_λ is the observed ellipticity in degrees at the wavelength λ, MRW is the average molecular weight of a residue, *l* is the pathlength of the cell in cm and *c* the concentration in g cm^{-3}.

Concentrated stock solutions were prepared by weighing the required quantity of polypeptide into a volumetric flask and dissolving it in the desired solvent. Solutions used for the measurements were prepared from these stock solutions.

Polarized i.r. spectra. I.r. spectra of oriented films of the polypeptides were obtained with a Perkin-Elmer 621 spectrophotometer, flushed with nitrogen (40 ft^3 min^{-1}). The oriented films were prepared according to the method of Ingwall *et al.*,[23] by stretching a poly(ethylene oxide) matrix containing the polypeptide.

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