Synthesis of Poly-(L-azetidine-2-carboxylic acid)

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Synthesis of poly-(L-azetidine-2-carboxylic acid) has been achieved by polymeric self-condensation of both the pentachlorophenyl and the succinimido-esters of L-azetidine-2-carboxylic acid hydrochloride. Racemization tests have been performed on model compounds and on polymer fractions with nearly identical molecular weights ($\bar{M}_w$) isolated from different preparations. The water content of the polymer has been determined from the water-binding isotherm at 23 °C.

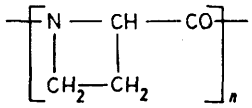
L-AZETIDINE-2-CARBOXYLIC ACID (Aze), a naturally occurring imino-acid homologous with proline, can be incorporated in place of proline into polypeptide chains of collagen and proteins.[1-5]

Replacement of proline generally results in drastic alterations of structures and biological functions. An inactive subunit of *E.coli* alkaline phosphatase is produced by substitution of Aze for proline.[6] The feeding of Aze to virus-infected cells inhibits the appearance of the small viral proteins which are normally formed from a very large polypeptide precursor.[7] The specific activity of the thyrotropin-releasing factor (TRF) is greatly depressed by substituting the prolineamide *C*-terminal residue with the closely related Aze–NH_2 group.[8] Growing chick embryos incorporate Aze into protocollagen with resulting inhibition of hydroxylation of the remaining prolyl residues, and marked decrease of the extrusion rate of collagen fibrils in the extracellular matrix.[9] The resulting accumulation of 'abnormal' collagen in chick embryo cells is accompanied by a morphological change in the connective tissues of the embryos.[9] A decrease of 1·8° in the melting temperature (T_m) for the helix–coil transition of the 'abnormal' collagen has been associated with a variation of the critical bond angles in the polypeptide chains, which destabilize the triple helix.[9]

The 'toxicity' of Aze residues has been explained with reference to the conformational difference between the four-membered ring of the azetidinyl residue and the five-membered ring of proline which significantly affects the secondary and tertiary structure of polypeptide chains.[5]

To elucidate the structural basis for these effects we are studying the conformational properties of a series of model oligo- and poly-peptides containing Aze residues, in solution and in the solid state.[10,11] A comparison with the already well-known conformational characteristics of the corresponding proline peptides and polypeptides may help to clarify how the conformation of the peptide chains might be affected when the azetidinyl residues replace proline.[11-18]

This paper describes the synthesis of a homopolymer



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(I), whose conformational properties in a variety of solvents have been already studied.[10,11]

The usual *N*-carboxy-anhydride (NCA) procedure for the synthesis of poly-(L-azetidine-2-carboxylic acid), $(Aze)_n$, cannot be followed because the *N*-chloroformyl-L-azetidine-2-carboxylic acid intermediate does not cyclize either by action of silver oxide in acetone or by addition of triethylamine *in situ*. Both methods have been successfully employed for the synthesis of L-proline NCA from the *N*-chloroformyl derivative obtained by treatment of L-proline with phosgene.[19,20] We have suggested that $(Aze)_n$ might be conveniently prepared by the polymeric self-condensation of the hydrochlorides of the imino-acid 'active esters' since the steric requirements which hinder the closure of the NCA ring on the pre-existing azetidinyl ring are also important for the competing reaction to *cyclo*(–Aze–Aze–).

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cyclo(–Aze–Aze–) can be prepared only in very low yields by cyclization of the linear dipeptide Aze–Aze–OMe. Experiments showed that *cis*-annulation of the two additional four-membered rings onto the piperazine-2,5-dione ring causes a change in the conformation of the central ring (from planar or nearly planar to a boat conformation) and a marked departure from planarity of the amide bonds.[21,22]

Satisfactory yields of polymers were obtained by polymerization of both the pentachlorophenyl and succinimido-esters of Aze prepared from their *N*-t-butoxycarbonyl derivatives.[23-29]

To obtain high-molecular weight homogeneous samples for conformational studies both polymers were separated from low molecular weight cyclo- and linear azetidinyl peptides by preparative gel filtration on

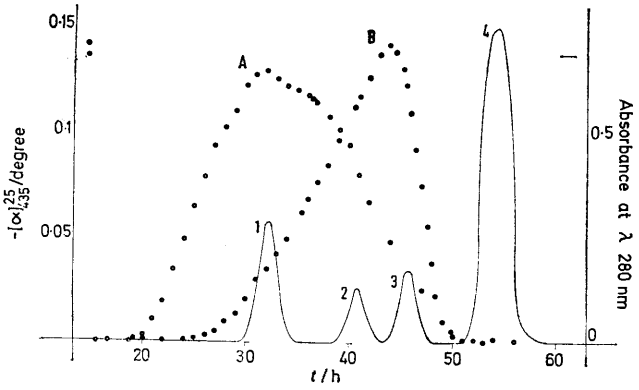

FIGURE 1 Elution profile (Bio-Gel A 5m) of poly-(L-azetidine-2-carboxylic acid) prefractionated *via* gel filtration on Sephadex G-50 (fine): A, sample obtained *via* pentachlorophenyl ester activation; B, sample obtained *via* succinimido-ester activation. Molecular weight calibrants: 1, myoglobin, mol. wt. = 17,800; 2, lime beam trypsin inhibitor, mol. wt. = 8400; 3, insulin B chain, mol. wt. = 3400; 4, L-tryptophan, mol. wt. = 204

Sephadex G-50 (fine) of the reaction mixture. Whereas activation with the pentachlorophenyl ester gives larger molecular weights, higher yields of polymer result from the polymerization of the succinimido-ester.

The molecular weights were estimated by agarose gel chromatography in 6M-guanidinium chloride by comparison of the elution peaks of the polymers with those of suitable polypeptide molecular weight markers (Figure 1) and then determined by ultracentrifugation in water according to the Yphantis midpoint method[30,31] (Figure 1).

The $\bar{M}_w$ values obtained by ultracentrifugation are 21,000 for the polymer prepared *via* the pentachlorophenyl ester and 5000 for that prepared *via* the succinimido-ester.

Elemental analyses of the samples recovered by lyophilization of the column eluates indicate a considerable amount of water of hydration in the polymer. We have performed experiments to determine accurately the water content of the polymers at different relative humidities and constant temperature by following the gravimetric method described by Gratton [32] (see Experimental section). At 23° and relative humidities between 30 and 60%, the water content of the polymers varies from 12 to 18%.

The optical purity of the polymer samples has been tested in view of the possible racemization of the activated monomers through direct α-hydrogen abstraction by triethylamine during the polycondensation.[33-35] We have found that the total acid hydrolysis method does not have the required accuracy since the azetidinyl residues decompose into several products in 6N-HCl at high temperature (~100°). The stereochemical purity of $(Aze)_n$ has therefore been evaluated by comparing the c.d. spectra of fractions with nearly identical $\bar{M}_w$ (8000) isolated from polymers which have been prepared *via* the foregoing two active esters under different polymerization conditions. The c.d. spectra are almost indistinguishable in water, trifluoroethanol, ethanol–water (99 : 1), and hexafluoropropan-2-ol.[11]

In a further racemization test, the specific rotations $[\alpha]_{589}^{25}$, of 2% (w/v) solution of Boc–Aze–OPcp and Boc–Aze–ONSu in dimethylformamide containing a 2 : 1 molar excess of triethylamine were recorded at regular intervals during 3 days. The optical activity remained identical with that measured immediately after dissolution of the samples.

These results and the close similarity of the c.d. patterns and molecular ellipticity values of $(Aze)_n$ and $(Pro)_n$ in solvents supporting the form I helix indicate that racemization through α-hydrogen abstraction does not occur significantly during the synthesis of $(Aze)_n$.

EXPERIMENTAL

L-Azetidine-2-carboxylic acid (Serva) was used as received, $[\alpha]_{589}^{22}$ −123·3° (*c* 3·6% in H_2O). The imino-acid has been also synthesized following the procedure of Rodebaugh and Cromwell,[36] $[\alpha]_{589}^{22}$ −123·5° (*c* 3·6% in H_2O) (Found: C, 47·5; H, 13·7; N, 7·0. Calc. for $C_4H_7NO_2$: C, 47·5; H, 13·85; N, 6·95%), *m/e* 101 (M^+).

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Triethylamine and dimethylformamide were purified by addition of *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester.[23] Dimethylformamide was tested with dinitrofluorobenzene for the presence of dimethylamine as described.[37]

Dioxan was dried and purified from peroxides by mixing with sodium methoxy(ethoxy)aluminium hydride (SDMA^R, ROC–RIC Organometallics). Ethyl acetate was dried over anhydrous $MgSO_4$ and fractionally distilled. All other solvents were of the highest obtainable purity. M.p.s were determined with a Tottoli apparatus, optical rotation with a Perkin-Elmer 141M automatic polarimeter in a standard 100 mm thermostatted cell, and n.m.r. spectra with a Varian T-60 spectrometer, with tetramethylsilane as internal standard. Mass spectra were determined with a Varian MAT III spectrometer operating at 80 eV: samples were introduced into the source by direct inlet system, probe temperature 80°.

Elemental analyses were carried out in the Laboratorio di Microbiologia, Snam Progetti S.p.A., Monterotondo, and the ultracentrifuge molecular weight determinations were performed at the Istituto Superiore di Sanità in a Spinco ultracentrifuge model E (Beckman Instruments). C.d. spectra were measured on a Cary 60 spectropolarimeter equipped with the model 6001 c.d. attachment. Solutions were prepared by weighing the samples into a volumetric flask and adding the solvent. Concentrations were calculated by allowing for the water content of the samples.

N-*t*-*Butoxycarbonyl*-L-*azetidine*-2-*carboxylic Acid* (Boc–Aze).—t-Butoxycarbonyl azide (10·8 ml, 87 mmol) was added to a solution of L-azetidine-2-carboxylic acid (7 g, 69·4 mmol) at pH 9·5 in water–dioxan (1 : 1; 30 ml) in the vessel of a pH-stat (Radiometer, Copenhagen) at room temperature.[38]

The NaOH uptake (4N-NaOH) was almost complete in 30 min, but the mixture was left at room temperature overnight until the turbidity completely disappeared. The solution was extracted thrice with ether. The aqueous phase was saturated with NaCl, chilled in ice, and then acidified to pH 3 with solid citric acid. The oil formed was extracted several times with ethyl acetate, and the organic solution washed with small portions of saturated NaCl solution, and dried ($MgSO_4$). Removal of the solvent gave an oil which was crystallized from ethyl acetate–hexane, yield 89%, m.p. 104—106°, $[\alpha]_{578}^{25}$ −124·6° (*c* 1% in ethyl acetate), δ ($CDCl_3$) 1·45 (9H, s, Bu^t), 2·11—2·77 (2H, complex, β-H_2), 3·74—4·13 (2H, t, γ-H_2), and 4·57—4·90 (1H, complex, α-H) (Found: C, 53·8; H, 7·45; N, 6·95. Calc. for $C_9H_{15}NO_4$: C, 53·75; H, 7·45; N, 6·95%) *m/e* 201 (M^+). The product showed only one spot by t.l.c. on silica gel H (Merck, AG) in the solvent systems: n-butanol–acetic acid–water (3 : 1 : 1); ethyl acetate–pyridine–acetic acid–water (60 : 20 : 6 : 14); and chloroform–benzene–acetic acid (85 : 10 : 5). The compound was located with ninhydrin after exposure to HCl fumes.

N-*t*-*Butoxycarbonyl*-L-*azetidine*-2-*carboxylic Acid Pentachlorophenyl Ester* (Boc–Aze–OPcp).—Freshly distilled *NN'*-dicyclohexylcarbodi-imide (10·32 g, 50 mmol) in ethyl acetate (24 ml) was added to a solution of pentachlorophenol (Fluka puriss., recrystallized from benzene; 13·3 g, 50 mmol) in ethyl acetate (190 ml) at 0°. The mixture was stirred at 0° for 7 min, then Boc–Aze (10·05 g, 50 mmol) in ethyl acetate (60 ml) was added and the reaction allowed to proceed for 1 h at the same temperature and for an additional hour after removal of the ice-bath.[39] *NN'*-Dicyclohexylurea was almost completely recovered by cooling the solution to −10°, and lowering the temperature to −20° after concentration to small volume. The crude ester, m.p. 95—96°, isolated by evaporation of the solvent, was recrystallized from absolute ethanol, yield 90%, m.p. 98—99°, $[\alpha]_{578}^{25}$ −72° (*c* 1% in ethyl acetate), δ ($CDCl_3$) 1·45 (9H, s, Bu^t), 2·27—2·93 (2H, complex, β-H_2), 3·87—4·33 (2H, complex, γ-H_2), and 4·80—5·23 (1H, complex, α-H) (Found: C, 40·25; H, 3·05; N, 3·05; Cl, 39·3. Calc. for $C_{15}H_{14}Cl_5NO_4$: C, 40·5; H, 3·1; Cl, 39·5; N, 3·1%). This compound was too unstable on silica gel for satisfactory t.l.c. analysis.

L-*Azetidine*-2-*carboxylic Acid Pentachlorophenyl Ester Hydrochloride* (Aze–OPcp,HCl).—Anhydrous HCl was bubbled for 45 min into a stirred solution of Boc–Aze–OPcp (10 g, 22·2 mmol) in anhydrous dioxan (400 ml) at 0°. The hydrochloride, which precipitated as a white crystalline solid during the reaction, was purified by repeated washing with anhydrous dioxan and ether, yield 80%, m.p. 134—135°, $[\alpha]_{578}^{25}$ −9·5° (*c* 1% in methanol), δ (CD_3OD) 2·93—3·30 (2H, complex, β-H_2), 3·98—4·35 (2H, complex, γ-H_2), and 5·63—5·84 (1H, complex, α-H) (Found: C, 31·0; H, 1·65; Cl, 54·8; N, 3·65. Calc. for $C_{10}H_7Cl_6NO_2$: C, 31·1; H, 1·8; Cl, 55·1; N, 3·6%.

N-*t*-*Butoxycarbonyl*-L-*azetidine*-2-*carboxylic Acid Succinimido-ester* (Boc–Aze–ONSu).—*N*-Hydroxysuccinimide (Emanuel) (1·71 g, 14·9 mmol) and Boc–Aze (3·0 g, 14·9 mmol) were dissolved in anhydrous 1,2-dimethoxyethane (15 ml) and cooled at 0°. After *ca.* 10 min, freshly distilled *NN'*-dicyclohexylcarbodi-imide (3·38 g, 16·4 mmol) in dimethoxyethane (15 ml) was added with stirring. The mixture was kept at 4° for 24 h.[24]

NN'-Dicyclohexylurea was filtered off and washed with small portions of cold dimethoxyethane. The solution was maintained for *ca.* 2 h at −20° for complete precipitation of *NN'*-dicyclohexylurea and then concentrated to small volume in a rotatory evaporator. Addition of n-hexane gave a slightly coloured powder, m.p. 114—116°, yield 93%. The ester was dissolved in warm ethyl acetate, decolourised on active charcoal, and recovered as a white crystalline powder by addition of n-hexane, yield 80%, m.p. 115—116°, $[\alpha]_{578}^{25}$ −117·2° (*c* 1% in ethyl acetate), *m/e* 298 (M^+), δ ($CDCl_3$) 1·45 (9H, s, Bu^t), 2·30—3·00 (2H, complex, β-CH_2), 2·83 [4H, s, CH_2CH_2(ONSu)], 3·80—4·23 (2H, complex, γ-H_2), and 4·77—5·07 (1H, complex, α-H) (Found: C, 52·4; H, 6·05; N, 9·3. Calc. for $C_{13}H_{18}N_2O_6$: C, 52·35; H, 6·05; N, 9·4%). This compound, like the other succinimido-esters, was too unstable on silica gel for satisfactory t.l.c., even in anhydrous solvents.

L-*Azetidine*-2-*carboxylic Acid Succinimido-ester Hydrochloride* (Aze–ONSu,HCl).—A solution of Boc–Aze–ONSu (1·3 g, 4·36 mmol) in anhydrous ethyl acetate (30 ml) was bubbled with dry HCl for *ca.* 1 h at *ca.* 15°. In the first stage of the reaction the hydrochloride separated as an oil, then precipitated as white crystals. The product was isolated by removing the solvent and washing the solid repeatedly with anhydrous ether. The hydrochloride is slightly hygroscopic and can be conveniently stored over KOH under vacuum, yield 75%, m.p. 127—129°, $[\alpha]_{578}^{25}$ −44·9° (*c* 1% in methanol), δ (CD_3OD) 2·70 [4H, s, CH_2CH_2-(ONSu)], 2·57—3·10 (2H, complex, β-H_2), 3·83—4·43 (2H,

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complex, γ-H_2), and 5·0—5·50 (1H, complex, α-H) (Found: C, 39·6; H, 4·71; Cl, 14·8; N, 11·5. Calc. for $C_8H_{11}ClN_2O_4$: C, 40·95; H, 4·7; Cl, 15·1; N, 11·95%).

Poly-(L-*azetidine-2-carboxylic acid*) via *the Pentachlorophenyl Ester.*—To a suspension of Aze–OPcp,HCl (1·16 g, 3 mmol) in anhydrous dimethylformamide (3 ml) anhydrous triethylamine (2 equiv., 0·83 ml) were added dropwise. Polymerization was allowed to proceed for 2 days at room temperature. The mixture was poured with stirring in a large excess of anhydrous ether and the precipitate was washed with several portions of ether. The product was finally recovered by centrifugation. Completion of the polyacylation reactions was demonstrated by the absence of the pentachlorophenyl ester peak in the i.r. spectrum at 5·58 μm.

Elimination of salts and low molecular weight contaminants was achieved by chromatography on Sephadex G-50 (fine) (90 × 3 cm; elution rate 40 ml h^{-1}). The column eluate was monitored at 220 nm by using a 0·025 cm flow-cell in a Cary 15 spectrophotometer. The yield of the polymer recovered by lyophilization of the column eluate was 26%. The polymer was rechromatographed on Sephadex G-50 (fine) at an elution rate of 25 ml min^{-1}. A fraction of $\bar{M}_w$ 21,000 was isolated in 10% yield, δ (D_2O) 2·04—3·05 (2H, complex, β-H_2), 3·94—4·57 (2H, complex, γ-H_2), and 4·87—5·12 (1H, complex, α-H).

Polymerization of Aze–OPcp,HCl was also carried out in anhydrous benzene at triethylamine : activated ester ratios of 1·0, 2·0, and 2·5. The yields and molecular weights of the resulting polymer are close to those obtained in dimethylformamide at identical Et_3N : activated ester ratios.

Poly-(L-*azetidine-2-carboxylic acid*) via *the Succinimido-ester.*—Aze–ONSu,HCl (1·1 g, 4·69 mmol) was suspended in anhydrous dimethylformamide (4·6 ml), and triethylamine (1·31 ml, 9·38 mmol) was added dropwise. A clear solution formed rapidly and then became increasingly cloudy. After 3 days at room temperature, the mixture was poured into an excess of ether and repeatedly washed with ether and methylene chloride. The crude polymer was dissolved in trifluoroethanol, filtered, and again recovered by evaporation to dryness under vacuum, yield of desalted, unfractionated polymer 40%. The polymer was purified from low molecular weight impurities by elution chromatography on Sephadex G-50 (fine) as described above, yield 26%. The ^1H n.m.r. spectrum in D_2O is identical with that of $(Aze)_n$ prepared *via* the pentachlorophenyl ester.

Molecular Weight Determination.—The molecular weights of polymers were estimated by chromatography on Bio-Gel A 5 m agarose gel (100—200 mesh) using columns equilibrated in 6M-guanidinium chloride. A 6M-solution of guanidinium chloride (Merck) in water was prepared by heating the salt to 60° in the presence of activated charcoal for 4 h and filtering through Whatman No. 42 filter paper. The column used was a Pharmacia chromatographic column (1·5 × 90 cm) which had been silanized before use with 5% (v/v) dichlorodimethylsilane–toluene at 60° for 2 h. The column was packed to a height of 85 cm with the agarose gel previously treated with 6M-guanidinium chloride (pH 5·0) and allowed to deaerate without stirring at room temperature for *ca.* 20 h. A solution (0·2 ml) containing the molecular weight markers myoglobin, lime beam trypsin inhibitor, insulin B chain, and L-tryptophan dissolved in 6M-guanidinium chloride (0·5 ml) at pH 5·0, was loaded on the top of the column and eluted at a flow rate of *ca.* 2·4 ml h^{-1}. The column effluent was passed through the flow-cell of a LKB Uvicord II (LKB Instruments, London) monitoring at 280 nm. After elution of the molecular weight calibrants, the polymers (6 mg in 0·5 ml guanidinium chloride) were applied to the column with elution conditions identical with those of the markers. The presence of the polymer in the fractions (collected in a Ultra Rac LKB 700 fraction collector) was detected by checking the optical rotation at 435 nm in a 10 cm micro-cell at 25°.

The $\bar{M}_w$ were also determined by equilibrium sedimentation ultracentrifugation. The ultracentrifugation measurements were performed at 25° in water. Concentrations in the range 0·5—1 mg ml^{-1} were used for the Yphantis runs. The rotor speed was 24,000 rev. min^{-1}. A partial specific volume, $\bar{v}$, of 0·756 ml mg^{-1} was determined in water by

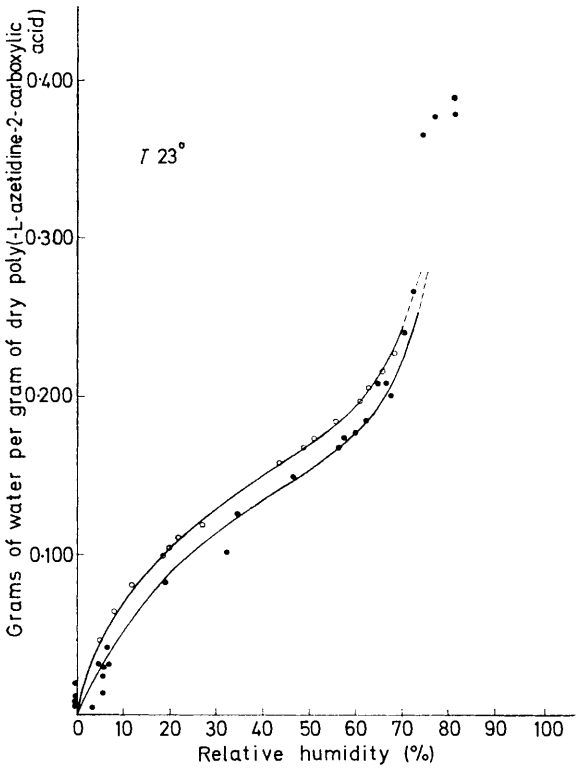

FIGURE 2 Adsorption (●)–desorption (○) of water on poly-(L-azetidine-2-carboxylic acid) at 23°

use of a digital high precision densitometer (DMA 02), as described by Kratky and his co-workers.[40]

Water Content Determination.—Samples of powdered polymer (*ca.* 5 mg) were placed on the plate of a Cahn electromicrobalance (Mod. RG) and kept for 24 h at 10^{-5} Torr and 23°. Dry, ultrapure nitrogen was gently passed through the vacuum chamber and the weight of the polymer determined. No further change of weight was observed by repeating the measurements for *ca.* 2 days. This weight was taken as the ' dry ' weight of the polymer. The samples were then hydrated by exposure to atmospheres of increasing relative humidities provided by a device in which ultrapure, dry nitrogen was mixed with water-saturated nitrogen. The relative humidities were continuously monitored by g.l.c. analysis of the hydrating gas by use of a Hewlett–Packard gas chromatograph HP 5712A (Poropak Q80—100

[40] O. Kratky, H. Leopold, and H. Stabinger, *Z. Angew. Phys.*, 1969, **27**, 273.

mesh column, 6 ft × ¼ in, maintained at 120°). The detector temperature was 200° and the carrier gas (helium) flow rate 100 ml min^{-1}. The increase in weight was automatically recorded during the hydration process.

Figure 2 shows the adsorption and desorption of water as a function of the relative humidity at 23°. An adsorption–desorption hysteresis is apparent.

Racemization Tests.—Boc–Aze–OPcp (0·204 g, 0·45 mmol) and triethylamine (0·126 ml, 0·9 mmol) were dissolved in dimethylformamide (10 ml) and placed in a polarimetric tube maintained at 25°. The optical rotations were measured immediately after sample dissolution and recorded repeatedly at regular intervals. The changes of $[\alpha]_{589}^{25}$ were less than the uncertainties in the measurements during 3 days, $[\alpha]_{589}^{25}$ −68·6° (c 2%).

Boc–Aze–ONSu (0·206 g, 0·69 mmol) and triethylamine (0·19 ml, 1·38 mmol) were dissolved in DMF and kept in a polarimetric tube. The optical activity was followed as above. A constant value of $[\alpha]_{589}^{25}$ −108° (*c* 2%) was found. The C.D. spectra of all fractions ($\bar{M}_w \simeq 8000$) isolated from different $(Aze)_n$ preparations in H_2O and fluorinated alcohols show a negative band centred at 216 nm, followed by an almost constant negative dichroism of low intensity in the wavelength range 208—188 nm. The molar ellipticity values at 216 nm are in the range from −13,000 to −13,400 deg. cm^2 $dmol^{-1}$. The c.d. spectra of $(Aze)_n$ fractions in ethanol–water (99 : 1; v/v) have a strong positive band at 219 nm and a negative, very intense maximum centred at 194 nm. Molar ellipticities in the ranges 88,000—89,000 and −129,000 to −133,000 deg. cm^2 $dmol^{-1}$ have been calculated from the spectra for all fractions at 219 and 194 nm, respectively.

Added in proof: An independent synthesis of Boc–L-azetidine-2-carboxylic acid has recently been described (A. M. Felix, M. H. Jimenez, R. Vergona, and M. R. Cohen, *Internat. J. Peptide and Protein Res.*, 1973, **5**, 201).

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