Synthesis and Circular Dichroism Characterization of L-Azetidine-2-Carboxylic Acid Cyclic Peptides*

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Synopsis

Several cyclic homopeptides containing L-azetidine-2-carboxylic acid (Aze)—an imino acid homologous with proline but containing one less methylene group in its cyclic side chain—have been prepared. The peptides reported include $cyclo(Aze)_2$, $cyclo(Aze)_3$, and $cyclo(Aze)_6$. The synthesis and spectral characterization of these cyclic peptides are described, and the results discussed in terms of the rigidity and steric constraints attributable to Azecontaining peptides. CD spectra of these materials in several solvents are reported and compared with those of proline analogs; the similarity between the CD spectra of $cyclo(Aze)_3$ and $cyclo(Pro)_3$ is noted.

INTRODUCTION

L-Azetidine-2-carboxylic acid (Aze) is a naturally occurring imino acid homologous with proline but containing one less side-chain methylene group. Using this Aze residue as a "probe,"^{1–5} whose distinctive features relative to proline are increased rigidity and reduced hydrophobicity of the side chain, we hope to clarify the structural factors which render the Pro residue so important in determining the conformation of peptide chains.⁶

Some recent results indicate that the solution conformational differences found among poly(Pro), poly(Aze), poly(Pro-Aze-Pro), and poly(Aze-Pro-Aze) are not clearly ascribable to the increased rigidity of the Aze ring but, rather, to different solvent interactions with the peptide groups of the chains. It has been suggested that these Aze-containing polymers assume the conformations of poly(Pro), but only in solvent media different from those in which the two forms of poly(Pro) are found.^{1,3,4}

Cyclic homopeptides of azetidine carboxyl acid constitute useful models for comparative studies with related Pro-containing peptides, since the high sequential symmetry of such compounds may allow an assessment of the

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relative importance of the various structural factors which determine the conformation of the entire molecule. In one series of investigations,⁷⁻⁹ Blaha and coworkers have compared the properties of a series of Pro-containing diketopiperazines with the corresponding Aze cyclic dipeptides [including cyclo(D-Aze-D-Aze)]. However, because of the particular steric constrictions of cyclic dipeptides, the conclusions derived from conformational studies on 2,5-piperazinediones may not always be generalized to larger cyclic peptides. For these reasons we have carried out the synthesis of several larger cyclic Aze-containing peptides.

Among cyclic proline-containing peptides, cyclo(Pro)₃ has been extensively studied, particularly in order to elucidate the details of its restricted conformation.^{10–14} Studies of the conformational mobility of proline residues in solution by measurements of ¹³C nmr spin-lattice relaxation times have found the pyrrolidine ring to be more flexible than previously thought, even in cyclo(Pro)₃.^{15–17} Thus, cyclo(Aze)₃, in which the side chain should be less flexible than in cyclo(Pro)₃, could be a useful model to aid in our understanding of the contribution of such factors as the rigidity of the side chain and the tendency toward peptide bond planarity in determining the molecular conformation. A schematic drawing of this molecule is presented in Fig. 1. In this, and the following communication,¹⁸ we report data on the synthesis and solution conformational properties of cyclo(Aze)₃ and related Aze-containing cyclohomopeptides.



Fig. 1. Schematic representation of cyclo(Aze)₃.

EXPERIMENTAL

Materials and Methods

The trifluoroacetic salt of the L-azetidine-2-carbonyl-L-azetidine-2carbonyl-L-azetidine-2-carboxylic acid pentachlorophenylester was prepared as previously described.³ L-Azetidine-2-carboxylic acid (Serva) was used as received. Purified and anhydrous dimethylformamide was obtained by addition of N-benzyloxycarbonylglycine p-nitrophenyl ester, followed by azeotropic distillation with benzene. Pyridine was dried by allowing it to stand over potassium hydroxide for several days and then heating it to reflux and fractionally distilling it from barium oxide. The mixed-bed ion-exchange resin used was AG-501-X8 (D) (H-OH), obtained from Bio-Rad Laboratories, Richmond, California. Sephadex G-15 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Mass spectra were determined with a Varian Mat III spectrometer operating at 80 eV: samples were introduced into the source by a direct inlet system. Elemental analyses were carried out in the Laboratorio di Microbiologia, Snamprogetti S.p.A., Monterotondo. Melting points were determined with a Perkin Elmer DSC-1B (differential scanning calorimeter) and optical rotations with a Perkin Elmer 141 M automatic polarimeter in a 100-mm thermostated microcell. Analytical determination of the L-azetidine-2-carboxylic acid methyl ester hydrochloride was performed on a Varian T-60 spectrometer with tetramethylsilane as the internal standard. Thin layer chromatography (TLC) was run on precoated silica gel F-254 and cellulose plates from Merck A.G., Darmstadt, Germany. The solvent systems used were *n*-butanol/acetic acid water/pyridine (15:3:12:10) (A) and methanol/chloroform (20:80) (B), all ratios by volume. The compounds were visualized with iodine.

Infrared spectra were obtained with a Perkin Elmer 325 spectrophotometer.

CD was measured on a Cary 60 spectropolarimeter equipped with the model 6001 CD attachment. The slit width was programmed to maintain a 15-Å half-band width; fused-quartz cells (0.001–0.01 cm) were used.

Cyclo(Aze)₃

The trifluoroacetic salt of the L-azetidine-2-carbonyl-L-azetidine-2carbonyl-L-azetidine-2-carboxylic acid pentachlorophenyl ester (2.8 g, 4.45 mmol), dissolved in dimethylformamide (85 ml), was added dropwise under nitrogen with efficient stirring over a 5-hr period to pyridine (2500 ml) at room temperature. Stirring was continued at room temperature for a week. After complete evaporation of the solvent, methanol was added and evaporated twice. To the residue (920 mg), after trituration and washings with ethyl ether, a minimum of methanol was added. Crystallization soon began, and 544 mg (four crops) of the cyclo(Aze)₃ was recovered by filtration.

Yield = 50%. ANAL: Calcd. for $C_{12}H_{15}N_3O_3$: C, 57.83; H, 6.02; N, 16.86. Found: C, 57.42; H, 6.00; N, 16.83. Physical constants of this peptide are given in Table I.

		Physi	ical Characteristics of Cyclic ((Aze) _n Peptides	-		
-	Melting	Mass	IR Be	ands	[\cv]258 [cv]578	Q	
Cyclic	Points ^a	Spectrometry	Carbonyl Ke	egion (cm ⁻¹)	C = 0.3%	IN I	
Peptides	(0°C)	(molecular ions)	KBr (Disks)	CH ₂ Cl ₂ (Sol.)	H ₂ O)	A	В
$Cyclo(Aze)_2$	207 - 212	m/e 166	1664(s), 1652(vs)	1676(sh), 1668(vs)	8.2	0.74	0.67
Cyclo(Aze) ₃	334 - 340	m/e 249	1658(vs), 1644(s),	1652(sh), 1647(vs)	20.8	0.70	0.80
			1628(vs), 1616(s)				
Cyclo(Aze) ₆	339-350	m/e 498	1679(vs), 1665(vs),	1679(vs), 1665(vs),	-352.0	0.17	0.46
			1640(sh)	1640(sh)			
^a Determined by (>310 dec.: cvclo(Az	differential scan e)e. >320 dec.	ming calorimetry; the m	elting points (uncorrected) det	ermined on a Kofler apparatus are	: cyclo(Aze) ₂	, 197–199; d	ycło(Aze) ₃ ,
^h Eluent $A = chlc$	proform/metha	nol (80:20), silica gel pla	ates; eluent $B = n$ -butanol/ace	etic acid/water/pyridine (15:13:12	:10), cellulose	e plates.	

TABLE I

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Cyclo(Aze)₆

The filtrate from which the cyclo(Aze)₃ crystallized was evaporated, and the residue (370 mg) was taken up in 30 ml of 50/50 (v/v) methanol/water mixture and stirred with the mixed-bed ion-exchange resin (15 g) for 1.5 hr at room temperature. The resin was removed by filtration, and the solvents were evaporated completely. The glasslike material surviving the resin treatment weighed 155 mg. A sample of 95 mg was dissolved in water and chromatographed on a Sephadex G-15 column. The column used was 200 cm long and had a diameter of 2.0 cm. The elution rate was 5.4 ml/ hr/cm². Prior to fraction collection, 240 ml of water were eluted. Then, fractions were collected for 20 min each. A total of 270 fractions were collected. Tubes were read at 230 nm. The elution profile showed four separate peaks (Fig. 2). The tubes from each peak were combined and lyophilized. From the first (tubes 143–150), second (tubes 153–165), third (tubes 179–200), and fourth (tubes 246–262) peaks, 4, 6, 50, and 9 mg were isolated, respectively. The main component (third peak, 50 mg) was $cyclo(Aze)_6$.

Calculated yield: 7%. ANAL: Calcd. for $C_{24}H_{30}N_6O_6$; $3H_2O$: C, 52.17; H, 6.52; N, 15.21. Found: C, 52.48; H, 6.16; N, 15.14. Physical constants of this peptide are given in Table I.

Cyclo(Aze)₂

The synthesis of the optically active azetidine-2-carboxylic acid anhydride was carried out following the procedure given in the literature for the racemic compound.¹⁹ We isolated and characterized the optically active azetidine-2-carboxylic acid methyl ester hydrochloride intermediate as described below. Thionyl chloride (3.88 mg, 0.032 mol) was added dropwise, over 30 min, to 15 ml methanol chilled to -10° C. After the addition of azetidine-2-carboxylic acid (3 g, 0.029 mol) into the solution, the mixture was stirred at -10° C for 1 hr. The temperature was then slowly raised to 40°C, and the mixture stirred at this temperature for an additional hour. After partial evaporation of methanol and excess thionyl chloride, the addition of ethyl ether gave an oily compound. The 2-carbomethoxyazetidine hydrochloride was isolated as a solid (mp = 75-77°C, 89% yield) by trituration with ethyl ether and drying over P2O5 in vacuo. Nmr spectrum (CDCl₃): "CH 5.3 (t), ⁷CH₂, 4.0–4.5 (complex), ^βCH₂, 2.5–3.1 (complex). The 2-carbomethoxyazetidine, obtained by the treatment of the hydrochloride with triethylamine, lost two molecules of methanol and formed crystals of $cyclo(Aze)_2$ (in nearly quantitative yield) upon standing at room temperature. Plates were obtained by recrystallization with acetone in 25% yield.

ANAL. Calcd. for $C_8H_{10}N_2O_2$: C, 57.83; H, 6.02; N, 16.86. Found: C, 57.22; H, 5.99; N, 16.73. Physical constants of this cyclic peptide are given in Table I.

Cyclo(Aze)₂ was also obtained from cyclo(Aze)₃ under the following



Fig. 2. Elution pattern of the cyclization reaction products of the linear precursor TFA-(Aze)₃-OPCP. c = 0.002M after previous isolation of cyclo(Aze)₃ and treatment of the residual solution with the mixed-bed resin which traps charged molecules. Sephadex G15 column dimensions: 200×2.0 cm; eluent: water; elution rate: 5.4 ml/hr/cm².

conditions: 50 mg cyclo(Aze)₃, dissolved in a mixture of H₂O/methanol 50:50 (v/v), were added to 15 g of mixed-bed resin and shaken for about 1.5 hr at room temperature. After filtration and washing, the product was isolated by evaporation of the solvent. TLC runs on the residue in solvent systems A and B showed that the main component of the mixture was cyclo(Aze)₂.

RESULTS AND DISCUSSION

The presence of both the *cis* and *trans* isomeric forms of the X-Aze peptide group was demonstrated by our recent solution conformational studies performed by CD, ¹³C, and ¹H-nmr spectroscopy on poly(Aze) and

FOF	Yield (%) (conc. <<0.01M)		Yield (%) (conc. = 0.01 <i>M</i>)	
Peptides	Cyclic Tripeptides	Cyclic Hexapeptides	Cyclic Tripeptides	Cyclic Hexapeptides
Pro-Pro-Pro ^b	83		60	
Sar-Pro-Pro ^b	28			38
Sar-Sar-Pro ^b	11		3	33
Sar-Sar-Sar ^c	16	25		38
Aze-Aze-Azed	50	7	30	10

TABLE II

^a Cyclization of the tripeptide pentachlorophenylester in pyridine.

^b See Ref. 21.

^c See Ref. 20.

^d This work.



Fig. 3. Elution patterns of cyclization reaction products at two different concentrations of TFA-(Aze)₃-OPCP [$c_1 = 0.002M$) (...); $c_2 = 0.01M$ (---)] after previous isolation of cyclo(Aze)₃ and treatment of the residual solution with the mixed-bed resin which traps charged molecules. Sephadex G15 column dimensions: 200×2.0 cm; eluent: water; elution rate: 3.0 ml/hr/cm².

on other L-azetidine-2-carboxylic acid derivatives.^{1,3,4} It was found that, as in the case of X-Pro peptide groups, the *cis* form is supported by less strongly interacting solvents.

We report here the synthesis of cyclo(Aze)₃ by ring closure at high dilution in pyridine of the linear tripeptide active ester. Carrying out the cyclization at room temperature and at a concentration in active ester of about 0.002M, we obtained a 50% yield of cyclo(Aze)₃, similar to that obtained by some of us for cyclo(Pro)₃,¹¹ but higher than that obtained in the case of cyclo(Sar)₃.²⁰



Fig. 4. CD spectra in methanol of $cyclo(Aze)_2 (\dots)$, $cyclo(Aze)_3 (\dots)$, and $cyclo(Aze)_6 (\dots)$.

As pointed out for the synthesis of cyclotripeptides, 10,21,22 factors affecting the yield of cyclization and the occurrence of higher cyclic products include: (1) the number of sites of possible *cis-trans* isomerism of the linear peptide, (2) the deviation from planarity of the peptide groups in the forming cyclopeptide, and (3) the side-chain rigidity.

	λ	m_{a}^{a}
Compounds	(nm)	(× 10 ⁻³)
$Cyclo(Pro)_2^{b}$	222	-5,300
	205	5,000
$Cyclo(Aze)_2$	230	-18,300
	209	31,800
Cyclo(Pro) ₃ ^b	250 (sh)	$\sim -5,000$
	233	-12,000
	215	19,000
	202	7,500
	190	30,000
Cyclo(Aze) ₃	257	-1,600
	232	-13,100
	217	18,500
	202	-15,200
	187	33,000
Cyclo(Aze) ₆	245 (sh)	~-1,500
•	218	-20,000
	198	22,300

TABLE IIICD Results in Methanol at 24°C

* See Ref. 11.

^b m_{θ} = mean residue ellipticity.

With $cyclo(Aze)_3$ the high cyclization yield suggests that the linear tripeptide has the all-*cis* conformation significantly populated among the allowed conformations in solution. An inspection of a Dreiding model of $cyclo(Aze)_3$, employing bond distances and angles derived from a recent study of the crystal and molecular structure of Boc-Aze,²³ suggests that the deviation from planarity of the peptide group in the cyclic molecule may be small but significant.

With Sar-Pro-Pro, Sar-Sar-Pro, and Sar-Sar-Sar, the cyclic hexapeptides are also formed, and the yield increases with the content of sarcosyl residues.²¹ This effect appears to correlate with increasing flexibility of the peptide chains. By contrast, it has been reported that $cyclo(Pro)_6$ is not formed at all, even in relatively concentrated solutions (0.1M).¹⁰ However, in the case of Aze-containing peptides, the cyclic hexapeptide is formed even at high dilutions ($\sim 0.002M$), although the yield remains low when the cyclization is carried out in more concentrated solution (0.01M), where cyclodimerization might be more favorable. In Table II the cyclization yields of $cyclo(Aze)_3$ and $cyclo(Aze)_6$ are shown at the two different concentrations and compared with those of $cyclo(Pro)_3$ and of sarcosyl-containing tripeptides and hexapeptides. Increasing the concentration of the linear tripeptide (to 0.01M) resulted in a decrease in the yield of cyclo(Aze)₃, a slight increase in the yield of $cyclo(Aze)_6$, and, interestingly, a significant formation of higher cyclic peptides, probably compounds such as cy $clo(Aze)_9$ and $cyclo(Aze)_{12}$ (Fig. 3). The apparent inability of linear homo-proline peptides to form cyclic peptides higher than $cyclo(Pro)_3$ may be related to the behavior observed for linear proline peptides in solution;



Fig. 5. CD spectra in methanol of $cyclo(Aze)_3$ (---) and $cyclo(Pro)_3$ (---).

i.e., these peptides adopt poly(Pro)II type all-*trans* conformations abruptly at five to six Pro residues.²⁴ Linear Aze peptides of this chain length and greater appear to retain the mixed *cis*-*trans* conformers,^{1,3,4} which are more conducive to cyclization (*vide infra*).

In Fig. 2, which represents the elution pattern of the reaction mixture after previous isolation of $cyclo(Aze)_3$ by crystallization and subsequent treatment of the residual solution with the mixed-bed resin, a peak appears



Fig. 6. CD spectra of cyclo(Aze)₃ in water (---) and H_2SO_4 (97%) (---): mol H⁺/mol Aze = 327.

at tubes 245–262 corresponding to cyclo(Aze)₂. The compound has been identified by TLC and mass spectrometry experiments. The cyclo(Aze)₂ apparently forms from the splitting of cyclo(Aze)₃ rings in the presence of the resin, as described in Experimental. The fact that the elution pattern

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of the crude reaction product (not shown) does not contain this peak demonstrated that, in our case, the cyclo(Aze)₂ is not formed during the cyclization reaction. In similar preparations of cyclic compounds, such as cyclo(Aze-Ala-Gly)₂ and cyclo(Gly-Aze-Gly)₂, treatment with the resin also leads to some hydrolysis of the cyclopeptides.²⁵ The use of the resin for the purification of the cyclic compounds from linear impurities should be viewed with caution until it can be established whether the resin could be responsible for the low yields of certain cyclic peptides described throughout the literature. A possible explanation for this behavior in the Aze cases is that "strained" (i.e., slightly nonplanar) X-Aze or Aze-Aze bonds in cyclic peptides are relatively susceptible to hydrolysis due to the increased electrophilic character expected for peptide carbonyl carbons of such bonds.

Figure 4 and Table III summarize the CD results in methanol of cy-



Fig. 8. CD spectra of cyclo(Aze)₆ in water (—) and H_2SO_4 (97%) (– –): mol H⁺/mol Aze = 214. The ellipticity values are calculated without taking into account water bound to the sample. The vertical bars, I, indicate the error limits of the measurements.

 $clo(Aze)_2$, $cyclo(Aze)_3$, and $cyclo(Aze)_6$, compared to those of the homologous proline compounds. In $cyclo(Aze)_2$ the enhanced intensity of the CD bands indicates that this molecule is conformationally more rigid than $cyclo(Pro)_2$. This effect originates from the decreased conformational mobility of the four-membered azetidine ring, in agreement with the nmr results.¹⁸ In the case of $cyclo(Aze)_3$ and $cyclo(Pro)_3$, the similarity of the CD patterns (Fig. 5) and the close intensity of the CD bands (except at 202 nm) suggest that the two molecules have similar conformations with respect to the basic structure of their nine-membered peptide backbone rings, although some flexibility of the azetidine ring has been detected by nmr experiments in water.¹⁸

In order to assign the CD bands of cyclo(Aze)₃, we have recorded spectra

in several solvents, e.g., water, methanol, tributylphosphate, sulfuric acid 97%, and dilute sulfuric acid. Pronounced red shifts of the CD band are observed in water (228 nm), in methanol (232 nm), and in tributylphosphate (235 nm), consistent with the expectation that bands due to $n \rightarrow \pi^*$ transitions shift to longer wavelength with decreasing solvent polarity. All the CD bands located in the wavelength range between 225 and 260 nm change position in H_2SO_4 (97%) (Fig. 6). These results suggest that the relatively large CD bands in the range 225-260 nm represent spectral manifestations of $n \to \pi^*$ electronic transitions.²⁶ The correctness of this assignment is strengthened by the following observations. By diluting a solution of cy $clo(Aze)_3$ in H₂SO₄ (97%) to a final ratio of H₂O/H₂SO₄ = 95/5 (v/v) and recording the CD spectrum very quickly (~ 5 min), the resulting pattern is very similar to that observed in pure water. In H_2SO_4 (97%) the CD pattern remains constant for at least 3 hr, excluding degradation of the molecule. Furthermore, in the CD spectrum of $cyclo(Aze)_2$ in H_2SO_4 (97%) (Fig. 7), the negative band observed in water at 227 nm is completely canceled out but appears again after dilution of H_2SO_4 with water to a ratio of $H_2O/H_2SO_4 = 95/5$ (v/v).

In the case of cyclo(Aze)₂, the molecule is stable in concentrated and dilute H_2SO_4 . As in cyclo(Aze)₃, we assign the CD band centered at 227 nm to the $n \rightarrow \pi^*$ amide transition. In the spectra reported in Figs. 4–8, the CD bands appearing below 225 nm can be assigned to the amide $\pi \rightarrow \pi^*$ transitions.

Figure 8 shows the CD spectra of $cyclo(Aze)_6$ in water and sulfuric acid. The small negative band appearing at 245 nm in water and as a shoulder at about 241 nm in methanol is attributed to the $n \rightarrow \pi^*$ transition, since it shifts in sulfuric acid. After dilution of the concentrated H₂SO₄ solution with water, the similarity of the CD spectrum with that recorded in water indicates that, as in the case of the other cyclic compounds, the molecule has not been degraded. The large differences in the CD spectra of cyclo(Aze)₆ in H₂O and methanol indicate that several stable conformers might exist, depending on the polarity of the solvents.

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