Acta Chemica Scandinavica B 30 (1976) 753—772

Cyclic Pentapeptides of Sarcosine in Combination with Either Alanine or Glycine. Syntheses and Conformational Processes Studied by NMR Spectroscopy

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A series of cyclic pentapeptides has been synthesised. Cyclic dipeptides, tetrapeptides and partially racemised tetra- and pentapeptides were isolated as by-products. The formation of the smaller rings is due to cleavage of the peptide chain during the cyclisation reaction.

NMR studies of the cyclic pentapeptides in $CHFCl_2$ showed that several conformational changes took place when solutions prepared by dissolving crystals at −70 °C were gradually heated to +20 °C. The conformers present in the final equilibrium, usually reached below room temperature, varied for the different cyclic pentapeptides and the initial crystal conformer was hardly present.

The driving force behind these conformational changes is suggested to be replacement of external hydrogen bonds in the crystal conformer with internal hydrogen bonds to form more stable conformers in solution. This requires conversion of *cis* amide bonds to *trans*.

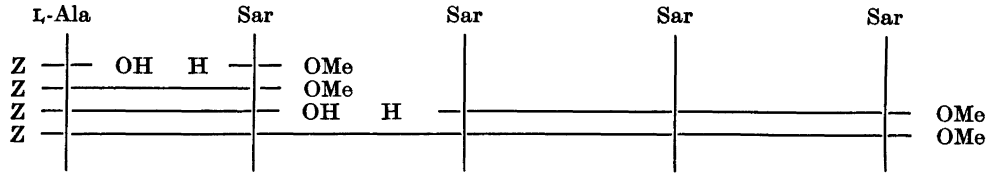
Attempts were made to distinguish between *cis* and *trans* amide bonds by the following NMR methods: differential solvent shifts, different band widths of the *N*-methyl lines, shifts induced by complexation with Eu, and shift differences in ^{13}C NMR, but no conclusive results could be obtained. Addition of benzene, was, however, helpful in resolving overlapping lines.

The majority of conformational studies on cyclic peptides have been carried out on cyclic dipeptides and cyclic hexapeptides, only a few isolated investigations have been reported for cyclic pentapeptides.[1] A difference was found in the yield on cyclisation of glycylleucyldiglycylleucine and of glycyldileucylglycylglycine and also higher yields when both D- and L-leucine was used when compared to the L,L-isomer.[2,3] Dielectric increment measurements showed a shorter distance between the ends of the linear peptides containing the L,D-form and this would favour the cyclisation. Five cyclic pentapeptides[4] such as cyclo-glycyl-L-alanyldiglycyl-L-prolyl and similar compounds were studied by NMR spectroscopy in dimethyl sulfoxide solution and found to be present in two conformations, about 70 % of the major and 30 % of the minor conformer. It was suggested that the major conformer contained only *trans* amide bonds whereas the minor conformer had the amide bond involving the proline nitrogen in the *cis* form. Both conformations contained intramolecular hydrogen bonds.

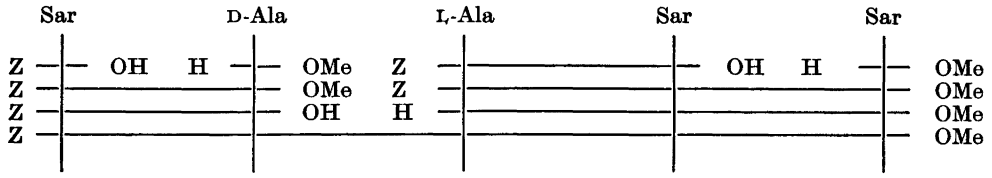
Our work with cyclic homologues of sarcosine[5,6] has shown that these cyclic peptides have a high degree of conformational homogeneity and most of the rings are present in solution in only one conformation. It was also found that a series of cyclic tetrapeptides[7,8] of sarcosine, in combination with one or two alanine or glycine residues, take the same conformation as the parent compound, cyclotetrasarcosyl. In these, the configuration of the four amide groups are alternately *cis* and *trans*. This skeleton was adopted even in those cases where an NH-amide had to take a *cis* configuration. The corresponding cyclic pentapeptide, cyclo-pentasarcosyl, when studied by NMR spectroscopy in deuteriochloroform, revealed one major and one minor (less than 10 %) conformation. Low-temperature NMR studies showed the major conformation to be the same as that in the crystal with the con-

Scheme 1. Routes to the different linear pentapeptides.

For cyclo-L-alanyltetrasarcosyl



For cyclo-D-alanyl-L-alanyltrisarcosyl



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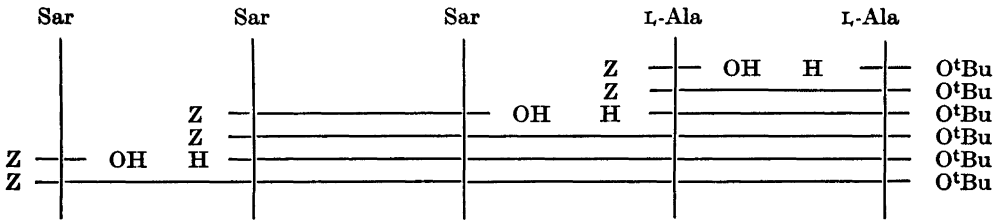
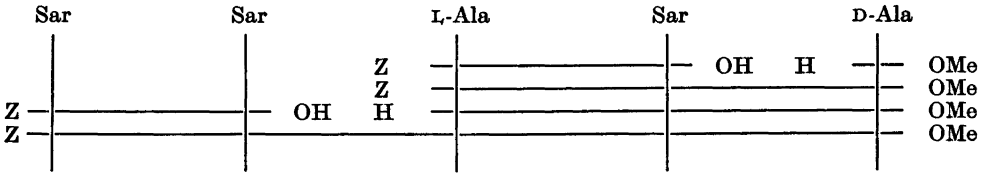
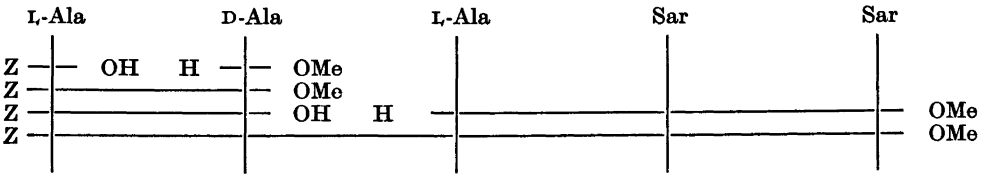


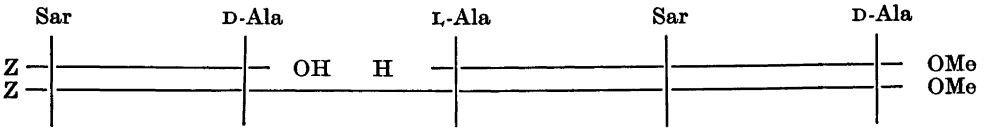
For cyclo-L-alanylsarcosyl-D-alanyldisarcosyl



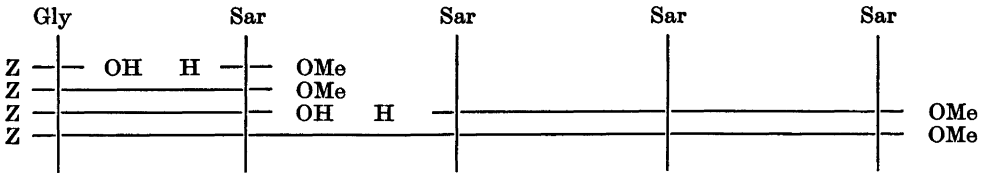
For cyclo-L-alanyl-D-alanyl-L-alanyldisarcosyl



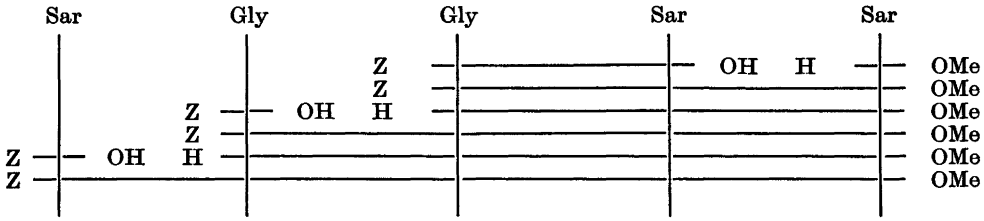
For cyclo-D-alanyl-L-alanylsarcosyl-D-alanylsarcosyl



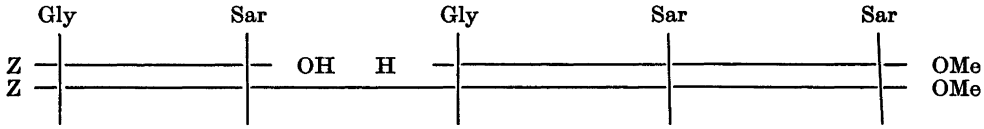
For cyclo-glycyltetrasarcosyl



For cyclo-diglycyltrisarcosyl



For cyclo-glycylsarcosylglycyldisarcosyl



figurational sequence *cis,cis,cis,trans,trans*.[9,10,6] It was therefore considered to be of interest to study a series of cyclic pentapeptides of sarcosine combined with alanine or glycine to see whether these possessed the same degree of conformational homogeneity as found for the analogous tetrapeptides. The compounds prepared and studied were six cyclic pentapeptides of sarcosine combined with alanine:
cyclo-L-alanyltetrasarcosyl (c-L-AlaSar$_4$), cyclo-D-alanyl-L-alanyltrisarcosyl (c-D-Ala-L-AlaSar$_3$), cyclo-di-L-alanyltrisarcosyl (c-L-Ala$_2$Sar$_3$), cyclo-L-alanylsarcosyl-D-alanyldisarcosyl (c-L-AlaSar-D-AlaSar$_2$), cyclo-L-alanyl-D-alanyl-L-alanyldisarcosyl (c-L-Ala-D-Ala-L-AlaSar$_2$), cyclo-D-alanyl-L-alanylsarcosyl-D-alanylsarcosyl (c-D-Ala-L-AlaSar-D-AlaSar),
and three cyclic pentapeptides of sarcosine in combination with glycine:
cyclo-glycyltetrasarcosyl (c-GlySar$_4$), cyclo-diglycyltrisarcosyl (c-Gly$_2$Sar$_3$), cyclo-glycylsarcosylglycyldisarcosyl (c-GlySarGlySar$_2$).

SYNTHESES

The different linear peptides were synthesised as shown in Scheme I. The benzyloxycarbonyl group was used for protection of the amino function, the methyl ester (in one case the *t*-butyl ester) for the carboxyl group and *N,N'*-dicyclohexylcarbodiimide as the coupling reagent. For cyclisation the methyl ester was hydrolysed by alkali (the *t*-butyl ester by trifluoroacetic acid) and the carboxyl group converted to the 2,4,5-trichlorophenyl ester, the benzyloxycarbonyl group was removed by hydrogenolysis and the peptide active ester cyclised in pyridine. After passage through a strong cat- and an-ion exchange column to remove noncyclised material, the cyclic peptides were purified by chromatography on silica gel, fractional crystallisation and sublimation. Not only the desired cyclic pentapeptide was isolated, but also dipeptides, tetrapeptides, decapeptides and racemised products (Table 1).

Formation of cyclic dipeptides was observed during cyclisation of peptides, especially those containing sarcosine as the second residue in the peptide chain. This was very prominent in a series of tetrapeptides [11] and was also observed in the series of homologous sarcosine peptides.[6] The free amino group attacks the second carbonyl carbon, with cleavage of the chain. Cycli-

sation of alanyltrisarcosylsarcosine trichlorophenyl ester gave together with the expected product two cyclic dipeptides, cyclo-alanylsarcosyl and cyclo-disarcosyl (Table 1). Here the cleavage of the peptide chain has occurred twice as was suggested to be the case in some of the sarcosine peptides.[6] However, from cyclisation of the closely related glycyltrisarcosylsarcosine only the corresponding cyclic pentapeptide was isolated. This shows that cleavage of the peptide chain occurs, but less generally in pentapeptides than in tetrapeptides.[11]

The formation of cyclic tetrapeptides was observed only in the cyclisation of disarcosyl-L-alanylsarcosyl-D-alanine trichlorophenyl ester where two cyclic tetrapeptides of sequence AlaSarAlaSar were isolated. These differed in solubility, sublimation temperature, IR and NMR spectra, and were assigned as cyclo-L-alanylsarcosyl-D-alanylsarcosyl (major product) and cyclo-L-alanylsarcosyl-L-alanylsarcosyl by comparison with the cyclic tetrapeptides previously studied.[8] The possibility that these arise by transannular reactions from the two cyclic pentapeptides formed (see below for racemisation) was excluded as these were recovered unchanged after boiling in pyridine for 9 h. Most probably these tetrapeptides were again formed by cleavage of the peptide chain, but as in both cases it is the *N*-terminal sarcosine which is lost, the attack must be from the carbonyl end. At first sight it is difficult to rationalise the preference of the carbonyl carbon of the second residue over the free amino group. However, the relatively low yield of cyclic pentapeptide and relatively high yield of cyclic tetrapeptide would suggest that the linear molecule exists in a conformation which favours the formation of the smaller ring. Cyclo-L-alanylsarcosyl-D-alanylsarcosyl is known from a previous study to take up the highly favoured *cis,trans,cis,trans* configuration with the C_α-methyl groups in favoured orientations.[8] This fits very well for folding the chain so that the amide nitrogen comes close to the active ester, and formation of a cyclic intermediate as shown in Fig. 1 is possible. The cleavage of the active ester may occur in several ways, either by the departure of a phenolate ion or elimination of phenol if the α-H of the D-alanine residue is removed. Subsequent loss of the *N*-terminal

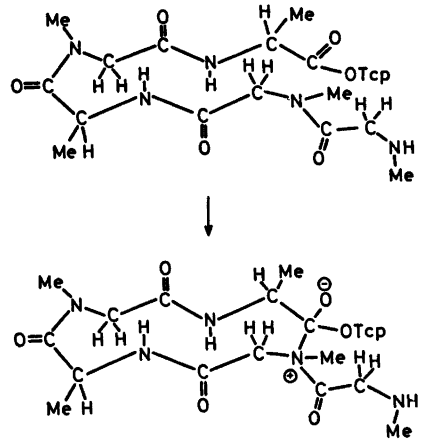


Fig. 1.

sarcosine unit leads to the two cylic tetrapeptides which are isolated on cyclising this specific pentapeptide.

Cyclic decapeptide formation can be very predominant from pentapeptides as shown in a systematic study on analogues of gramicidin S.[12–14] Earlier studies with homologous sarcosine peptides [6] have shown that this doubling reaction is not very dominant even on cyclisation of disarcosylsarcosine, and a similar situation has now been observed for the pentapeptide series. In most cases the presence of the cyclic decapeptide could only be inferred from the TLC. Attempts to isolate these small quantities did not succeed as they showed no tendency for crystallisation and were bound strongly to silica gel. Only for alanyltrisarcosylsarcosine was the cyclic decapeptide isolated by precipitation.

Racemisation was shown to have occurred by the isolation of pairs of pentapeptides (and tetrapeptides) from some of the reactions. Thus disarcosyl-L-alanylsarcosyl-D-alanine trichlorophenyl ester gave two cyclic pentapeptides (identical *m/e* 355) which had different melting points and different R_F values on TLC and could be separated on silica gel. A similar situation was found on cyclising sarcosyl-D-alanyl-L-alanylsarcosyl-D-alanine trichlorophenyl ester where two cyclic pentapeptides were isolated with different melting points and R_F values. As the two linear pentapeptides both contain D-alanine as the C-terminal amino acid it is likely that on cyclisation in the basic

medium at the elevated temperature (115 °C) some racemisation of the C-terminal amino acid takes place. The two cyclic pentapeptides formed in the former case, are therefore most likely cyclo-L-alanylsarcosyl-D-alanyldisarcosyl, m.p. 274 °C (major product) and cyclo-L-alanylsarcosyl-L-alanyldisarcosyl, m.p. 224 °C. This is in good agreement with the two cyclic tetrapeptides which also were formed in this cyclisation. The products in the second case have most likely an analogous relationship: cyclo-D-alanyl-L-alanylsarcosyl-D-alanylsarcosyl, m.p. (subl.) 317 °C (major product), and cyclo-D-alanyl-L-alanylsarcosyl-L-alanylsarcosyl, m.p. 260 °C. From each of the other cyclisation reactions only one cyclic pentapeptide was isolated, even though the X-ray analysis of the expected cyclo-L-alanyltetrasarcosyl showed that the crystal contained the racemate.[15] It is likely that the racemate crystallised more easily than each of the optical antipodes and that the crude cyclised product is not fully racemised. The optical rotation is low, but this can depend on the conformations of the cyclic peptides. The same was noted on crystallising the analogous cyclic tetrapeptide cyclo-L-alanyltrisarcosyl where the racemate was found in the crystal structure.[16] Thus with both the penta- and the tetrapeptide partial racemisation has taken place at some stage during the reaction sequence. Recently, it has been reported that racemisation does occur in reactions which had been thought to be racemisation free.[17–19]

PHYSICAL AND SPECTRAL PROPERTIES OF THE CYCLIC PENTAPEPTIDES

In contrast to the cyclic oligomers of sarcosine which have sharp melting points,[6] several of the cyclic pentapeptides, after being isolated by column chromatography, melted over a wide range. However, the melting points became sharper after recrystallisation from a suitable solvent (*e.g.* acetone) by slow evaporation in contact with the air. The infrared spectra, showing broad absorptions, gave little information. The mass spectra showed the molecular ions as intense peaks and very similar fragmentation patterns. The NMR spectra were generally more complex than in the sarcosine series but again gave the most informative picture of the conformational situation. When dissolved in $CDCl_3$ at room temperature, the number of lines showed the presence of two or more conformations in equilibrium. The conformational interconversion barriers are high, as temperatures of around 100 °C are needed to obtain coalescence to a single set of lines. This means that at low temperatures (about −70 °C) the conformers, at least as defined by a given *cis,trans*-sequence, will have life-times of hours. It should therefore be feasible to observe the conformation present in the crystal when crystals are dissolved at low temperature (−70 °C) and the NMR spectrum recorded at this temperature.* Slow warming of the sample in the NMR tube to room temperature and recording of the NMR spectra will reveal if any conformational changes take place.

When the initially isolated cyclic pentapeptides of broad melting range were dissolved at −70 °C, the NMR spectra were complex and showed that several conformations were present, while the sharply melting crystals when dissolved at −70 °C showed only one conformer (called A) at this temperature (Figs. 2–5). On allowing the latter samples to warm slowly to about −30 °C, new lines due to a second conformer (called B) began to appear. On further warming a third conformer (C) developed whilst the concentration of the crystal conformation A diminished. The full thermodynamic equilibrium, normally reached already below room temperature, consisted of a mixture of B and C and in some cases a fourth conformer D. Only in a few cases was the crystal conformer present at room temperature. The changes are most easily seen in the *N*-methyl region, the methylene protons, however, do not often give well resolved lines, but in those cases where the quartets are recognized the coupling constants are grouped around 14 and 18 Hz indicating that the CC_α-torsional angles take up the *anti*, *gauche* pattern as seen for the oligopeptides of sarcosine.[6] The NH protons could not be ob-

* Rotation about the single bonds is not expected to be slowed down sufficiently at these temperatures, but in a series of related cyclic peptides, cyclic oligomers of sarcosine, both the CC_α- and the NC_α-torsion angles take up only a limited number of values. The CC_α-torsion angles are either 180 or 60° and the NC_α-torsion angles are grouped around 90°.

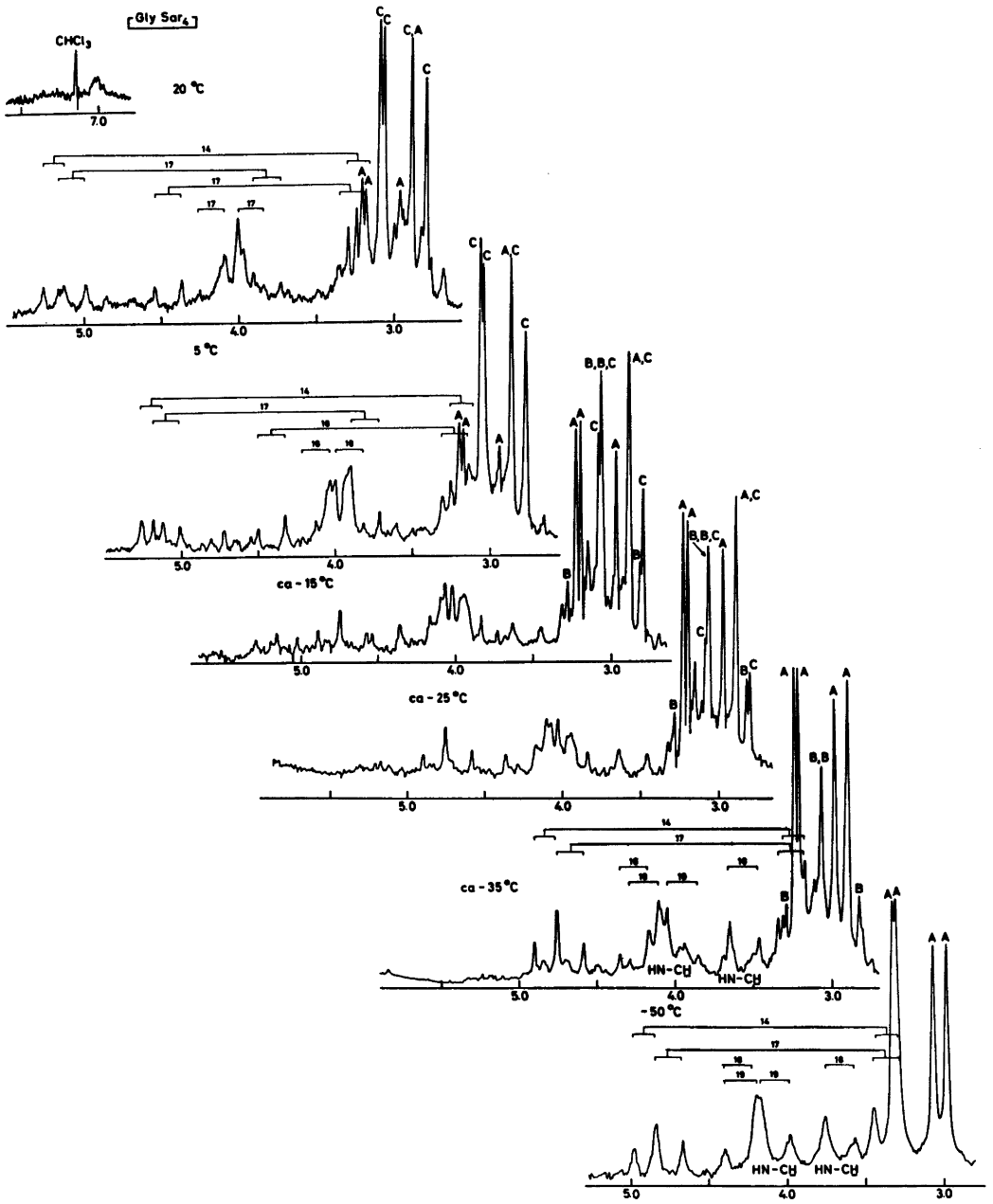


Fig. 2. The 100 MHz NMR spectrum of c-GlySar$_4$. Crystals were dissolved in $CHFCl_2$ at −50 °C (lower spectrum) and the solution allowed to warm up in the probe (NH-region left out). $CDCl_3$ was used as the solvent at the highest temperature (20 °C).

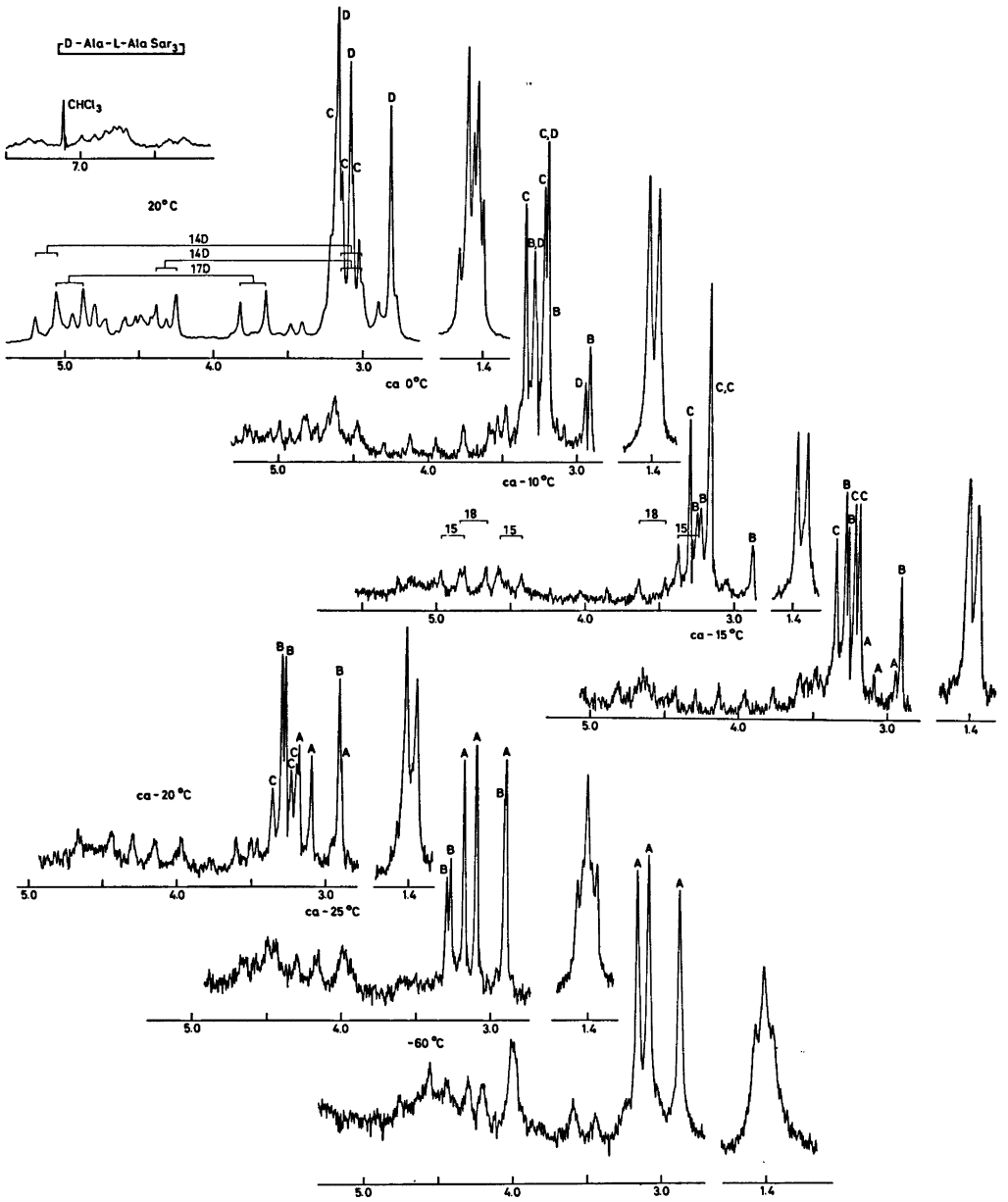


Fig. 3. The 100 NMR spectrum of c-D-Ala-L-Ala-Sar_3. Crystals were dissolved in $CHFCl_2$ plus traces of TFA at -60 °C (lower spectrum) and the solution allowed to warm up in the probe (NH-region left out), $CDCl_3$ was used as the solvent at the highest temperature (30 °C).

served due to the chemical shifts of the $CHFCl_2$ proton, the solvent used in these NMR studies, either pure or together with a small amount of trifluoracetic acid to increase the solubility. When studied in deuteriochloroform at room temperature the NH protons are close to the chloroform peak and the spin-spin coupling to the methine protons, which are hardly resolved, varies from 1 to 10 Hz.

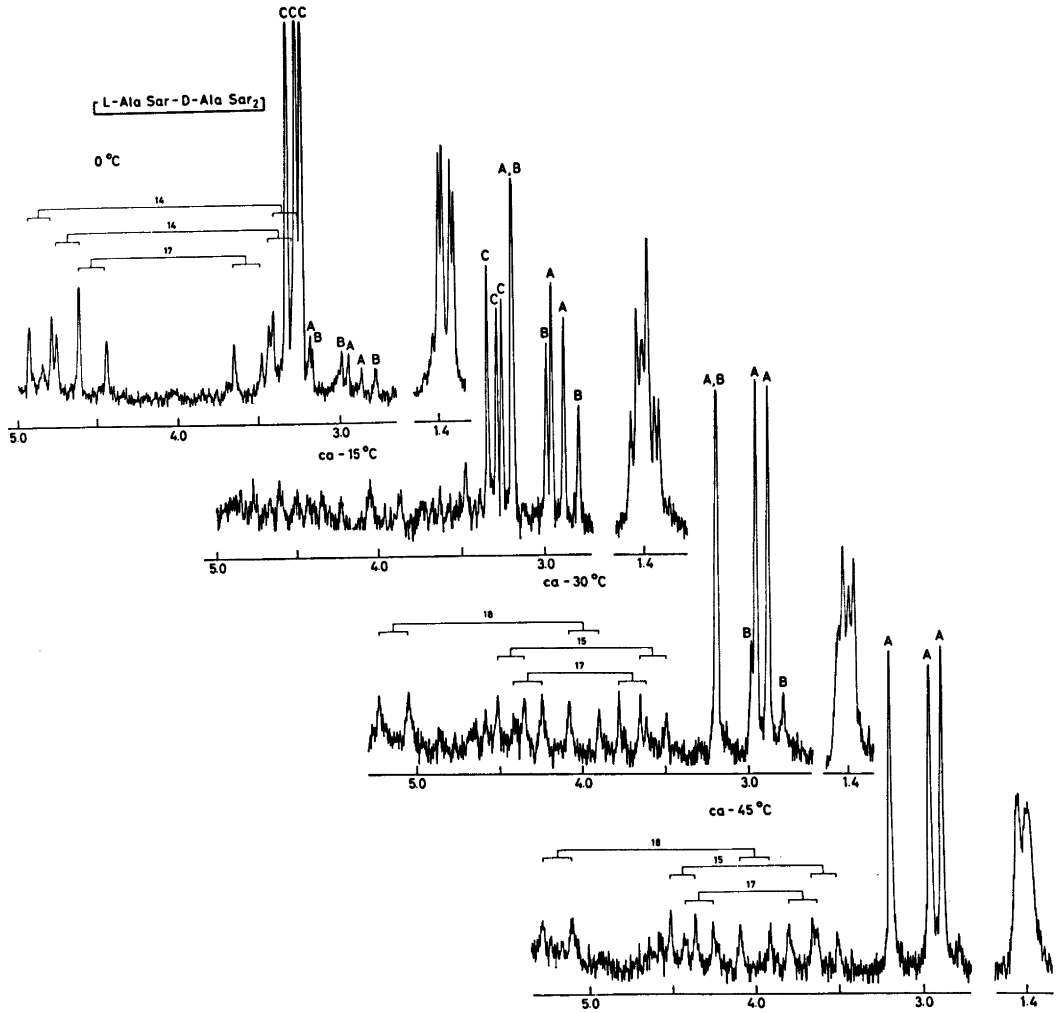


Fig. 4. The 100 MHz NMR spectrum of c-L-AlaSar-D-AlaSar$_2$. Crystals were dissolved in $CHFCl_2$ at −45 °C (lower spectrum) and the solution allowed to warm up in the probe (NH-region left out).

The conformational changes of the individual cyclic pentapeptides are closely related, but the concentration of the transient conformers differ and also the conformers present at equilibrium. c-GlySar$_4$ (Fig. 2) shows when dissolved at −50 °C four N-CH_3 lines due to the presence of one conformer, A. The CH_2-quartets are partly resolved. At about −35 °C a second conformer B appears, its concentration is always less than that of A and decreases when a third conformer C appears. At room temperature the conformer mixture is dominated by C (80 %) with a small amount of A but hardly any of B. This equilibrium mixture was warmed further to observe coalescence phenomena. The CH_2-quartets of conformer C started to broaden at 30 °C, at about the same temperature (40 °C) as was found [6] for c-Sar$_5$. Thus conformer C possesses the same high barrier to ring inversion as found in c-Sar$_5$.

c-AlaSar$_4$ (Fig. in Ref. 32) shows at −60 °C four N-CH_3 lines, but the C-CH_3 is split into two doublets. This indicates the presence of two conformers having N-CH_3 groups of iden-

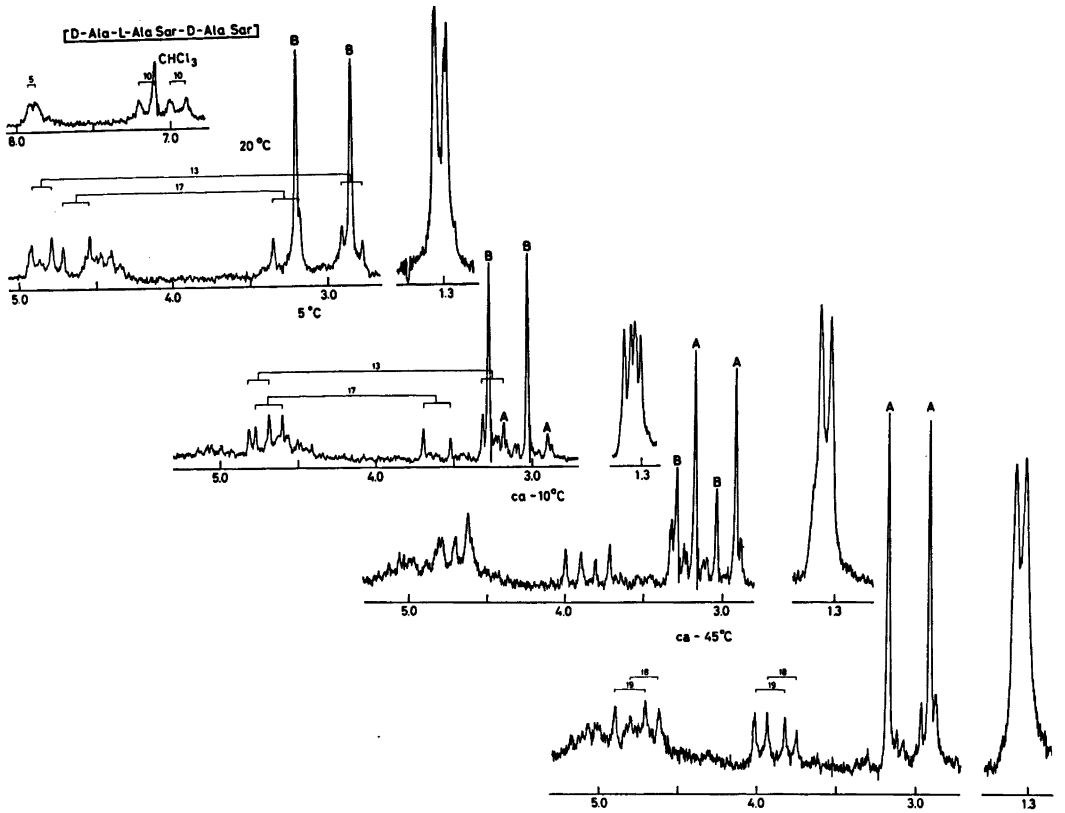


Fig. 5. The 100 MHz NMR spectrum of c-D-Ala-L-AlaSar-D-AlaSar. Crystals were dissolved in $CHFCl_2$ plus traces of TFA at −45 °C (lower spectrum) and the solution allowed to warm up in the probe (NH-region left out). $CDCl_3$ was used as the solvent at the highest temperature (20 °C).

tical chemical shifts. One of the conformers may represent the crystal conformation whereas the other may occupy the same ring skeleton, but with the *C*-CH_3-group inverted. The new set of four *N*-CH_3 lines due to conformer B becomes in this molecule equal to A in concentration before conformer C appears, and at room temperature C dominates (60 %), together with B (40 %), but only traces of A are present.

c-D-Ala-L-$AlaSar_3$ (Fig. 3) and c-Gly_2Sar_3 exhibit identical conformational changes, conformer B approaching A in concentration before C increases to become the dominating conformer. At about 0 °C a fourth conformer D develops and at room temperature the mixture consists of mostly D (70 %) and some C (30 %). The closely related c-L-Ala_2Sar_3 is the only compound in the series which shows a complex low temperature spectrum with several conformers present. On warming the sample, the picture became even more complex.

c-L-AlaSar-D-$AlaSar_2$ (Fig. 4) and c-L-AlaSar-L-$AlaSar_2$ show identical NMR spectral changes with rising temperature. Conformer A has three sharp *N*-CH_3 signals and three well resolved CH_2-quartets. B does not here approach A in concentration before the third conformer C appears. C increases to become the only visible conformation (> 90 %) at room temperature. This indicates that the conformational transformations of these molecules go through the same ring conformations, and that the C_α-methyl groups derived from the C-terminal alanine (D or L) have coincident chemical shifts. Both c-GlySarGlySar_2 and c-L-Ala-D-Ala-L-$AlaSar_2$ undergo analogous conformational changes with C as the dominating conformer in the final equilibrium mixture.

Conformer A of c-L-Ala-D-Ala-L-AlaSar$_2$ has two sharp N-CH_3 lines and two resolved CH_2-quartets both with J of 18 Hz and conformer C has two narrow split N-CH_3 lines and two well resolved CH_2-quartets with J of 18 and 14 Hz. The CH_2-quartets of c-GlySarGlySar$_2$ are not resolved and started to coalesce at *ca.* −5 °C.

c-D-Ala-L-AlaSar-D-AlaSar (Fig. 5) shows at low temperature two N-CH_3 lines and two CH_2-quartets. In this case no conformational change takes place before at about −15 °C when a second conformer B develops. On further warming, A diminishes as B increases in concentration, but no third conformer C appears, and at room temperature pure B is present. This is the only compound in the series where only one conformational transformation is observed. The closely related c-D-Ala-L-Ala-Sar-L-AlaSar shows, however, a different conformational behavior. One major conformation A is present at low temperature. The second conformer B becomes equal to A in concentration before the third conformer C develops at about −30 °C. In the equilibrium mixture there is hardly any B while A and C are present in equal amounts. These compounds do not adopt the same ring skeletons nor do the same conformational transformations occur, although the only difference is that one α-CH_3 group in one is inverted to the other. Steric interaction of the three α-methyl groups may stabilise different conformations.

ATTEMPTS TO APPLY NMR METHODS FOR DISTINGUISHING BETWEEN *cis* AND *trans* AMIDE IN CYCLIC PEPTIDES

In order to understand the conformational changes it was important to know if *cis-trans* amide interchange was involved. Some attempts were therefore made to investigate if it might be possible to distinguish between the N-CH_3 of *cis* amide bonds and those of *trans* in the sarcosine peptides. Several methods have been used to distinguish between *cis* and *trans* amides, such as differential solvent shifts, different band width of the N-methyl lines, shifts induced by complexation with paramagnetic metal ion and shift differences in ^{13}C NMR. These effects have been discussed for simple amides and small peptides. The N-methyl groups in N,N-dimethylformamide [20] and related compounds differ in chemical shift, being highest for the methyl group *cis* to the carbonyl due to the anisotropy of this group. A similar difference is observed in N-acetylsarcosine methyl ester [21] and in N-acetyl-N-methyl-L-alanine methyl ester [22] where the N-methyl protons of the preferred *trans* conformation are shifted to lower field than the *cis* N-methyl protons. This assignment has been extended to polysarcosine [21] where the conclusion is drawn that both *cis* and *trans* amides are present and to helical poly-N-methyl-L-alanine which shows only *trans* amides.[22] In an aromatic solvent, like benzene, the N-methyl peaks are shifted upfield, but to different extents due to a specific solvent/solute interaction. The N-methyl *trans* to the carbonyl shows a greater upfield shift than that *cis* to the oxygen.[23] This effect appears to be general for simple amide systems as it is observed in several N,N-dialkylamides,[24] some small, medium and large sized N-methyllactams [25] and in small peptides such as N-acetyl-N-methyl-L-alanine methyl ester.[26] In addition to chemical shift differences a broadening of the N-methyl peaks *cis* to the carbonyl is observed due to a small long range spin coupling through the amide bond to the C_α-protons.[27] This coupling is largest when the N-methyl group is *anti* to a C_α-proton. In a series of dipeptides with N-methylated amino acid as the second residue the N-methyl peak which belongs to *cis* amides is broader than that of *trans* amides.[28] The different effects described for simple flexible systems were applied to our cyclic sarcosine peptides which differ firstly in being relatively small rings with several amide groups present and secondly in being rigid systems. The most suitable molecule with which to check these techniques was cyclo-tetrasarcosyl where the NMR parameters are known. Cyclo-tetrasarcosyl is a centrosymmetric molecule with the amide sequence alternately *cis* and *trans*. Replacement of one sarcosine residue with alanine [7] showed that the upfield N-methyl peak belongs to a *trans* amide group in contrast to what is usually found in the simple amide systems. Furthermore, the upfield peak of the N-methyl *trans* to the carbonyl is significantly broader and exhibits a small coupling of about 0.5 Hz. Spin decoupling showed that this is

Table 1. Yields (%) of cyclic peptides from pentapeptide trichlorophenyl ester.

Starting material	Dipeptides	Tetrapeptides	Pentapeptides	Decapeptides
HCl.H-AlaSar$_4$-OTcp	c-Sar$_2$ (20 %) c-AlaSar (14 %)		c-AlaSar$_4$ (46 %)	c-(AlaSar$_4$)$_2$ (3 %)
HCl.H-Sar-D-Ala-L-AlaSar$_2$-OTcp			c-D-Ala-L-AlaSar$_3$ (38 %)	c-(D-Ala-L-AlaSar$_3$)$_2$ (traces)
HCl.H-Sar$_3$-L-Ala$_2$-OTcp			c-L-Ala$_2$Sar$_3$ (36 %)	c-(L-Ala$_2$Sar$_3$)$_2$ (traces)
HCl.H-Sar$_2$-L-AlaSar-D-Ala-OTcp	c-Sar$_2$ (10 %)	c-L-AlaSar-D-AlaSar (11 %) c-L-AlaSar-L-AlaSar (3 %)	c-L-AlaSar-D-AlaSar$_2$ (11 %) c-L-Ala-Sar-L-AlaSar$_2$ (6 %)	
HCl.H-L-Ala-D-Ala-L-AlaSar$_2$-OTcp			c-L-Ala-D-Ala-L-AlaSar$_2$ (28 %)	c-(L-Ala-D-Ala-L-AlaSar$_2$)$_2$ (traces)
HCl.H-Sar-D-Ala-L-AlaSar-D-Ala-OTcp			c-D-Ala-L-AlaSar-D-AlaSar (55 %) c-D-Ala-L-AlaSar-L-AlaSar (5 %)	
HCl.H-GlySar$_4$-OTcp			c-GlySar$_4$ (53 %)	c-(GlySar$_4$)$_2$ (traces)
HCl.H-SarGly$_2$Sar$_2$-OTcp			c-Gly$_2$Sar$_3$ (6 %)	
HCl.H-GlySarGlySar$_2$-OTcp	c-GlySar (8 %)		c-GlySarGlySar$_2$ (4 %)	

Table 2. The chemical shifts of the NCH_3 protons of cyclic peptides in $CDCl_3$.

	$(NCH_3)_1$		$(NCH_3)_2$		$(NCH_3)_3$		$(NCH_3)_4$		$(NCH_3)_5$		ml C_6D_6 added to 0.5 ml $CDCl_3$
	δ	$\Delta\delta$(Hz)[a]	δ	$\Delta\delta$(Hz)	δ	$\delta\Delta$(Hz)	δ	$\Delta\delta$(Hz)	δ	$\Delta\delta$(Hz)	
c-Sar$_4$	3.0	(19)*cis*	3.0	(19)*cis*	2.83	(36)*trans*	2.83	(36)*trans*			0.4
c-Sar$_5$	3.14	(49)	3.01	(36)	2.93	(4)	2.93	(2)	2.91	(18)	0.4
c-Sar$_6$	2.96	(20)	2.96	(35)	2.91	(10)	2.91	(31)			0.35
c-(Sar$_3$OGl)$_2$	2.98	(30)	2.92	(13)	2.90	(56)					0.35
c-AlaSar$_4$(B)	3.30	(58)	3.25	(49)	3.12	(57)	2.83	(21)			1.2
c-AlaSar$_4$(C)	3.18	(80)	3.07	(105)	2.92	(23)	2.68	(38)			1.2
c-D-Ala-L-AlaSar$_3$ (C)	3.20	(52)	3.15	(30)	3.07	(47)					0.8
c-D-Ala-L-AlaSar$_3$ (D)	3.17	(41)	3.10	(27)	2.84	(16)					0.8
c-L-AlaSar-D-AlaSar$_2$ (C)	3.25	(27)	3.16	(54)	3.11	(27)					0.5
c-L-Ala-D-Ala-L-AlaSar$_2$ (C)	3.26	(51)	3.11	(64)							0.8
c-D-Ala-L-AlaSar-D-AlaSar (B)	3.2	(68)	2.85	(22)							0.9
c-GlySar$_4$ (C)	3.10	(59)	3.09	(90)	2.89	(26)	2.81	(35)			0.8

[a] $\Delta\delta$ represents the chemical shift differences in Hz when the given quantity of C_6D_6 is added in chloroform. The letters B, C and D represent the different conformations formed from the crystal conformation. OGl stands for the glycolic acid residue, $-OCH_2CO-$.

due to coupling to one of the C_α-protons adjacent to the nitrogen, that one which is *anti* to the *N*-methyl group (across a w-path). No coupling through the amide bonds was observed. The structure [7] shows none of the C_α-protons across the amide bonds to be *anti* to the *N*-methyl groups which had been observed to give the largest coupling constant.[28] Dropwise addition of benzene moved the peaks upfield, the *trans* *N*-methyl more than the *cis* (Table 2), which is in accord with the results from the simple amides. These effects were further checked using the higher cyclic sarcosine homologues with known conformation. Cyclo-pentasarcosyl which contains two *trans* and three *cis* amide bonds shows in chloroform solution two "low field" *N*-methyl peaks and three at higher field. On addition of benzene the former moved upfield to a greater extent than the latter indicating that these low field *N*-methyl peaks belong to the two *trans* amides. Of the other three signals two are virtually unaffected by the benzene addition and one shifts slightly upfield (Table 2). Cyclo-octasarcosyl with the amide sequence *cis,cis,trans,trans, cis,cis,trans,trans* and two-fold symmetry in the molecule has two different *trans* and two different *cis* *N*-methyl groups. In the NMR spectrum in chloroform solution two *N*-methyl peaks are resolved, but on addition of benzene four peaks appear. One of these is shifted slightly upfield, one significantly upfield, and the remaining two are intermediate. The same situation was found in the related depsipeptide, c-$Sar_3OGlSar_3OGl$,[29] which takes the same conformation but with one *trans* *N*-methyl peak lacking (Table 2). Peak broadening was difficult to observe in the larger molecules mostly due to the complexity of the spectra. This shows that solvent effects which seem to be general for open chain peptides cannot be extended to cyclic peptides. The folding of the chain into a ring may lead to situations where the chemical shift of an *N*-methyl group is influenced also by carbonyl groups further along the chain but close in space.

Lanthanide shift reagents which induce pseudocontact shifts by interaction with the lone pair of the carbonyl oxygen, usually shift a methyl group *cis* to the carbonyl more downfield than one being *trans*.[30] When $Eu(fod)_3$ was added to a chloroform solution of cyclotetrasarcosyl, the *cis* *N*-methyl group was shifted more than *trans*, but in cyclo-pentasarcosyl one of the two "low-field" *N*-methyl signals assigned to *trans*, showed the largest shift together with one of the three high-field signals, assigned to *cis*. The other signals were also shifted, but to a lesser extent. These downfield shifts are not consistent with the upfield shifts found in benzene, but as the Eu-complex, when situated at a given carbonyl group, will to a greater extent than benzene influence all protons of the molecule, it will be difficult to predict the different shifts when several amide groups are involved.

Carbon-13 magnetic resonance has been used to distinguish between *cis* and *trans* amides in peptides where the nitrogen of proline is part of the peptide bond.[31] Distinct differences in chemical shifts especially for the γ-carbon in the proline ring were found such that the minor *cis* isomer was shifted about 2 ppm upfield relative to the *trans* isomer and that the chemical shifts were the same for several compounds of this type. ^{13}C resonance spectra of cyclo-tetrasarcosyl, cyclo-alanyltrisarcosyl and cyclo-pentasarcosyl, for which the conformations are known, and of cyclo-glycyltetrasarcosyl were recorded at 15 MHz in chloroform solution. Cyclo-tetrasarcosyl showed *cis* and *trans* peaks, separated by 2 – 4 ppm for each of the three types of carbon atoms. Cyclo-alanyltrisarcosyl showed four carbonyl carbons where two belong to *cis* amide bonds and two to *trans*, but these did not form two distinct groups, the *N*-CH_3 *cis* and *trans* were separated by only 1 ppm. In cyclo-pentasarcosyl four of the five carbonyl carbons were resolved, but again not in two groups and the *N*-CH_3's were hardly resolved. These preliminary ^{13}C investigations showed no clear differences in the chemical shifts due to *cis* and *trans* amide bonds. The conformationally unknown cyclo-glycyltetrasarcosyl showed a similar picture with no well separated *cis-trans* peaks.

Thus, none of the different NMR methods used to distinguish between *cis* and *trans* amide bonds in small linear peptides showed convincing results when applied to the cyclic homologues of sarcosine and derivatives. Although the benzene addition showed some evidence that the *N*-CH_3 of a *trans* amide group

moves more upfield than N-CH_3 of a *cis* amide group, the difference in values can be small and cannot be relied upon diagnostically. Benzene addition to the cyclic pentapeptides was, however, useful to resolve overlapping signals since the conformation does not change on changing solvent. Table 2 shows the chemical shifts of the N-methyl protons in $CDCl_3$ solution and the upfield $\Delta\delta$ shifts when C_6D_6 is added mainly for the conformations B and C (D). Addition of benzene was also attempted at low temperature to the A-conformation but with little success due to the difficulty in keeping the temperature low enough so that the B and C conformers did not develop.

DISCUSSION

The NMR spectra clearly show that in these cyclic pentapeptides the crystal conformations are not the same as those favoured in solution. The conformational transformations might well go through additional transient unpopulated conformers not visible in the NMR spectra, but this seems hardly likely and will not be further considered. Conformational transformations seem to be prominent for cyclic pentapeptides containing NH-amide groups. Only the parent compound, c-Sar_5, retains the same conformation in solution as in the crystal.[6] Replacement of one or more sarcosine units with NH-amino acids as glycine or alanine lead to a mixture of conformations in solution with the crystal conformation hardly present. The ability of the NH-amides to form hydrogen bonds has already been proposed as the driving force for these conformational changes.[32] External hydrogen bonds stabilise the crystal conformers while intramolecular hydrogen bonds stabilise the conformers in solution, and the conformational changes must involve stepwise transformations of *cis* amide bonds to *trans*. Among the NH containing cyclic pentapeptides only c-$AlaSar_4$ [15] (Fig. 6A) has had its crystal conformation determined by X-ray methods. It is found to be identical to that of c-Sar_5,[10] both having the amide sequence *cis,cis,cis,trans,trans* (NH). The NH-amide group which occupies one of the two *trans* positions makes an external hydrogen bond with the carbonyl oxygen of the other *trans* amide group of a neighbouring molecule. On dissolution, the intermolecular hydrogen bonds are lost and the new conformer B may be favoured by a hydrogen bond across the ring. This is only possible if one of the *cis* amide groups becomes *trans*, and the sequence *cis,cis,trans,trans,trans* (NH) is suggested [32] (Fig. 6B). The more stable conformer C may arise by transformation of yet another *cis* amide bond to *trans*, to the sequence *trans,cis, trans,trans,trans* (NH) (Fig. 6C). Both B and C resemble the conformations found by X-ray studies of c-Gly_6 [33] and c-Ala_2Gly_4 [34] which contain both inter- and intramolecular hydrogen bonds.

It is likely that all cyclic pentapeptides which can easily fit the crystal conformation of c-$AlaSar_4$, without having any NH-amide group *cis*, will adopt this skeleton in the crystal. Thus, c-$GlySar_4$ may take this skeleton with the NH-amide in one of the two *trans* positions, but not necessarily the same position as in c-$AlaSar_4$ since the α-substituent is now lacking. The same conformational transformations may take place, but in this case conformer B is less stable compared to C as the mixture at equilibrium hardly contains any

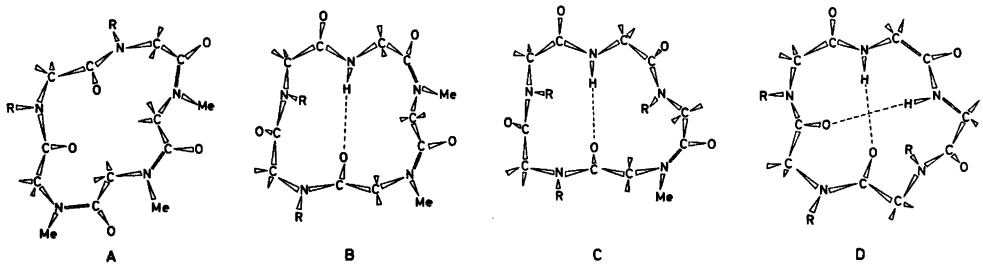


Fig. 6.

B. Both c-D-Ala-L-AlaSar$_3$ and c-Gly$_2$Sar$_3$ fit the crystal skeleton of c-AlaSar$_4$ with the two NH-amides in the *trans* positions, and the same conformers B and C can be formed also here. The last transformation to D seems less clear but may well correspond to the conversion of the remaining *cis* amide bond to *trans*, giving a conformation with all amide bonds *trans* as the most stable. Two intramolecular hydrogen bonds can then be formed as shown in Fig. 6D which is just one example among several possibilities for internal hydrogen bonding.

c-L-AlaSar-D-AlaSar$_3$ and c-L-AlaSar-L-AlaSar$_3$ which have identical NMR spectra both at low and high temperatures cannot easily adopt the crystal conformation found for c-AlaSar$_4$ which would require one NH-amide bond to be *cis*. A crystal structure of a related molecule, actinomycin, which has two identical cyclic pentapeptides, (16-membered ring) shows the amide sequence *cis,cis,trans,trans,trans* with the nitrogens of proline and sarcosine as part of the *cis* amide bonds and a strong hydrogen bond between the pentapeptides.[35] Two adjacent *cis* amide bonds are also found in some sarcosine peptides[6] and it seems therefore likely that the crystal conformation of c-L-AlaSar-D-AlaSar$_3$ has the amide sequence *cis,cis,trans* (NH), *trans,trans* (NH), the same sequence as suggested for conformer B of c-AlaSar$_4$, and with one intra- and one intermolecular hydrogen bond. The conformational transformations may occur as for c-AlaSar$_4$ where the conformer C (Fig. 6C) corresponds to conformer B in c-L-AlaSar-D-AlaSar$_3$ with the amide sequence *trans,cis,trans* (NH), *trans*, *trans* (NH) and one intramolecular hydrogen bond. The second transformation leads to conformer C which should have all the amide bonds *trans* and be similar to that in Fig. 6D. c-GlySarGlySar$_3$ and c-L-Ala-D-Ala-L-AlaSar$_3$ can go through the same conformational transformations and take the same ring conformations without ever having any NH amide bond in *cis*, and the NMR spectra which show closely related changes support this picture.

c-D-Ala-L-AlaSar-D-AlaSar which undergoes only one conformational change from A to B (Fig. 5) cannot easily adopt any of the two aforementioned skeletons for the crystal conformation, but is believed to have a ring conformation close to that in Fig. 6C with the amide sequence *cis,trans* (NH), *trans* (NH), *trans*, *trans* (NH). The transformation of conformer A to B may then correspond to a conversion of the remaining *cis* amide bond to *trans* (Fig. 6D). c-D-Ala-L-AlaSar-L-AlaSar which shows two conformational changes may have the amide sequence *cis,trans* (NH), *trans* (NH), *cis*, *trans* (NH) for conformer A. B may originate by transformation of one of the two *cis* amide bonds to *trans*, giving the sequence *trans,trans* (NH), *trans* (NH), *cis,trans* (NH) and further transformation of the last *cis* amide bond leading to conformer C.

In this series of cyclic pentapeptides a conformation with all amide groups *trans* seems to be the most stable in chloroform solution as the number of observed transformations corresponds to the initial number of *cis* amide bonds present. The only exceptions are c-AlaSar$_4$ and c-GlySar$_4$ which can form only one intramolecular hydrogen bond. Of course, these arguments imply that the barrier for each of the successive transformations is higher than the preceding one.

More definite conclusions about these conformational changes and the nature of the different conformers which are formed must await further detailed NMR studies (^{13}C) and more X-ray structural studies.

EXPERIMENTAL

Solvents used in the reactions were of analytical grade, the light petroleum had b.p. 40 – 60 °C. The monobeded ion-exchange resin, Amberlite, MB-1, analytical grade, was used, and the eluent was methanol. The fully protected linear peptides could all be prepared in methylene chloride. After removal of the benzyloxycarbonyl group was the residual peptide ester (confirmed by NMR) used immediately on isolation.

Many of the peptide derivatives were either viscous oils or non-crystalline solids. Their identity and purity were confirmed by NMR spectroscopy (Varian 60 A) and thin-layer chromatography performed on silica gel G in various solvent systems: ethyl acetate – chloroform 1:2 (A), 5 % methanol – chloroform (B), 10 % methanol – chloroform (C), 15 % methanol – chloroform (D), 20 % methanol – chloroform (E), acetic acid – methanol – chloroform 2:8:90 (F), acetic acid – methanol – chloroform 5:15:80 (G), acetic acid – water – ethanol 10:10:80 (H).

Samples for elemental analysis of the Z-peptide esters were purified on a silica gel column eluting with chloroform, otherwise stated. For the pentapeptides the chloroform elution was followed by chloroform added 2 % methanol, and for the cyclic peptides 2 – 10 % methanol was added to the chloroform. The tendency for the cyclic pentapeptides to contain water even after extensive drying as shown by NMR spectroscopy rendered elemental analysis relatively uninformative.

Abbreviations: Et_3N = triethylamine, DCC = dicyclohexylcarbodiimide, HOTcp = 2,4,5-trichlorophenol, DMF = *N*,*N*-dimethylformamide.

Cyclo-L-alanyltetrasarcosyl

General method for preparation of benzyloxycarbonyl-peptide ester (Z-Sar_2-OMe).[6]

Z-L-AlaSar-OMe. Sarcosine methyl ester hydrochloride[36] (35 g = 250 mmol) was suspended in CH_2Cl_2 (500 ml), Et_3N (25.5 g = 250 mmol) added and the solution filtered before combined with benzyloxycarbonyl-L-alanine[37] (56 g = 250 mmol) in CH_2Cl_2 (100 ml), cooled to – 15 °C and DCC (53.5 g = 260 mmol) added in portions. The reactions mixture was allowed to attain room temperature during 15 h and worked up as described.[6] The residual oil was dissolved in ether and precipitated with light petroleum to leave a viscous oil (72 g = 93 %), homogeneous by TLC, R_F 0.5 (A).

General method for preparation of the benzyloxycarbonyl-peptide acids (Z-Sar_2-OH).[6]

Z-L-AlaSar-OH. Z-L-AlaSar-OMe (70 g = 227 mmol) was hydrolysed in 1 N NaOH (230 ml) for 2 h and the mixture concentrated to ca. 40 ml and extracted twice with CH_2Cl_2. The aqueous layer was then acidified, extracted with CH_2Cl_2, washed with water and dried. The resultant oil was treated with ether and light petroleum and crystallised from acetone on addition of ether (56 g = 84 %), TLC, R_F 0.5 (F), m.p. 137 °C, $[\alpha]_D^{22}$ – 26.3° (*c* 2, MeOH). (Found: C 57.17; H 6.33; N 9.66. $C_{14}H_{18}N_2O_5$ requires C 57.13; H 6.17; N 9.52).

Z-L-AlaSar$_4$-OMe. Z-L-AlaSar-OH (6.5 g = 22 mmol), H-Sar_3-OMe[6] (5.3 g = 21.6 mmol) and DCC (5 g = 24.2 mmol) in CH_2Cl_2 (100 ml) resulted in a white solid (9 g = 80 %). TLC, R_F 0.45 (C), m.p. 65 °C, $[\alpha]_D^{22}$ – 5.7° (*c* 2, MeOH). (Found: C 54.95; H 6.75; N 13.46. $C_{24}H_{35}N_5O_8$ requires C 55.26; H 6.76; N 13.43).

Z-L-AlaSar$_4$-OH. Z-L-AlaSar$_4$-OMe (7.8 g = 15 mmol) and NaOH (16 ml) was reacted for 2 h. After acidification the acid was extracted into chloroform, dried and evaporated. The resulting peptide acid (7 g) was purified by extraction from a CH_2Cl_2 solution into an alkaline aqueous layer, acidification with 2 N hydrochloric acid and extraction into chloroform, followed by drying and evaporation. This afforded a white powder (6.7 g = 88 %), TLC, R_F 0.45 (G), softened at 95 °C, $[\alpha]_D^{22}$ – 5.9° (*c* 2, MeOH). (Found: C 54.42; H 6.49; N 13.76. $C_{23}H_{33}N_5O_8$ requires C 54.43; H 6.55; N 13.80).

General method for preparation of benzyloxycarbonyl-peptide 2,4,5-trichlorophenyl ester (Z-Sar_3-OTcp).[6]

Z-L-AlaSar$_4$-OTcp. Z-L-AlaSar$_4$-OH (3.7 g = 7.3 mmol), HOTcp (2.2 g = 11.1 mmol) and DCC (1.6 g = 7.7 mmol) in CH_2Cl_2 (50 ml) gave a white solid (4.5 g = 90 %), TLC, R_F 0.6 (C). An analytical sample (0.3 g) was further purified on a small silica gel column, softened at 75 °C. (Found: C 50.98; H 5.05. $C_{29}H_{34}N_5O_8Cl_3$ requires C 50.70; H 4.99).

General method for preparation of cyclic peptides (c-Sar_3).[6]

Cyclisation. Z-L-AlaSar$_4$-OTcp (1 g = 1.4 mmol) was hydrogenated for 50 min in methanol (80 ml) containing conc. HCl (0.1 ml) and 5 % Pd-C (0.3 g). The resulting yellowish semi solid (0.8 g = 1.36 mmol = 94 %) of HCl.H-L-AlaSar$_4$-OTcp was dissolved in DMF (50 ml) and added dropwise to stirred pyridine (500 ml) at 115 °C over a period of 1 h and stirred for another 1.5 h. After evaporation the residue was dissolved in methanol (100 ml), passed through an ion-exchange column and eluted with methanol. The first eluate (200 ml) contained most of the cyclic peptides. This was evaporated to dryness, treated with acetone (5 ml) and the undissolved material cyclo-alanyltetrasarcosyl filtered off. The filtrate was combined with the remaining column eluate (400 – 600 ml), which contained further cyclic peptides together with some DC-urea, this total solution evaporated to dryness and purified on a silica gel column, eluting with chloroform followed by chloroform-methanol (2 – 10 %). Yields of cyclic compounds: Cyclo-alanyltetrasarcosyl (45.5 %), TLC, R_F 0.45 (D), m.p. 245 – 250 °C, *m/e* 355, $[\alpha]_D^{22}$ – 2.3° (*c* 2, MeOH), cyclo-disarcosyl (20 %), cyclo-alanyl-sarcosyl (14 %), cyclo-alanyltetrasarcosyl-alanyltetrasarcosyl (3 %), m.p. 195 °C, *m/e* 710. The cyclo-alanyltetrasarcosylalanyltetrasarcosyl was not isolable on the column but was isolated by precipitation from methanol-ether.

Cyclo-D-alanyl-L-alanyltrisarcosyl

Z-Sar-D-Ala-OMe. D-Alanine methyl ester hydrochloride[38] (14 g = 100 mmol), Et_3N (10.5 g = 104 mmol), benzyloxycarbonylsarcosine (22.7 g = 102 mmol) and DCC (22 g = 107 mmol) gave a semi solid (27 g = 87 %), TLC, R_F 0.5 (A). A sample was purified. (Found: C 58.63; H 6.67; N 8.90. $C_{15}H_{20}N_2O_5$ requires C 58.43; H 6.54; N 9.09).

Z-Sar-D-Ala-OH. Z-Sar-D-Ala-OMe (13 g = 42.2 mmol) and NaOH (43 ml) (1.5 h) gave a solid, recrystallised from acetone (10.6 g = 86 %), TLC, R_F 0.55 (F), m.p. 115 – 118 °C, $[\alpha]_D^{22}$ – 6.1° (*c* 2, MeOH). (Found: C 57.15;

H 5.70; N 9.38. $C_{14}H_{18}N_2O_5$ requires C 57.13; H 6.17; N 9.52).

Z-L-AlaSar$_2$-OMe. Sarcosine methyl ester hydrochloride (15 g = 108 mmol), Et_3N (11 g = 109 mmol), Z-L-AlaSar-OH (30 g = 102 mmol) and DDC (23 g = 110 mmol) gave an oil (34 g = 88 %). TLC, R_F 0.5 (C).

Z-Sar-D-Ala-L-AlaSar$_2$OMe. Z-Sar-D-Ala-OH (10 g = 34 mmol), H-L-AlaSar$_2$-OMe (8.5 g = 34.7 mmol) [obtained by hydrogenation of Z-L-AlaSar$_2$-OMe (13.5 g = 35.6 mmol)], DCC (7.7 g = 37.4 mmol) resulted in a foamy solid (15.2 g = 86 %), TLC, R_F 0.5 (C). An analytical sample softened at 70 °C, $[\alpha]_D^{22}$ +18.2° (*c* 2, MeOH). (Found: C 55.05; H 6.83; N 13.66. $C_{24}H_{35}N_5O_8$ requires C 55.26; H 6.76; N 13.43).

Z-Sar-D-Ala-L-AlaSar$_2$-OH. Z-Sar-D-Ala-L-AlaSar$_2$-OMe (11 g = 21 mmol) and NaOH (22 ml) (2 h) resulted in a solid, recrystallised from acetone (9.3 g = 87 %), TLC, R_F 0.5 (G), softened at 90 °C, $[\alpha]_D^{22}$ +5° (*c* 2, MeOH). (Found: C 54.37; H 6.50; N 14.08. $C_{23}H_{33}N_5O_8$ requires C 54.43; H 6.55; N 13.80).

Z-Sar-D-Ala-L-AlaSar$_2$-OTcp. Z-Sar-D-Ala-L-AlaSar$_2$-OH (5 g = 9.86 mmol), HOTcp (2.8 g = 14.2 mmol) and DCC (2.2 g = 10.7 mmol) gave a semi-solid (5.4 g = 80 %), TLC, R_F 0.55 (C). An analytical sample softened at 70 °C. (Found: C 50.76; H 5.22. $C_{29}H_{34}N_5O_8Cl_3$ requires C 50.70; H 4.99).

Cyclisation. Z-Sar-D-Ala-L-AlaSar$_2$-OTcp (1.2 g = 1.75 mmol) was hydrogenated and the white solid (0.9 g = 1.53 mmol = 88 %) of HCl.H-Sar-D-Ala-L-AlaSar$_2$-OTcp cyclised and worked up as described earlier. The residue after passage through an ion-exchange column was taken into small amount of acetone and undissolved material filtered off; a further precipitate formed when kept at 0 °C overnight. The solid consisted mainly of cyclo-D-alanyl-L-alanyltrisarcosyl with some DC-urea which was removed by sublimation (heated to 210 °C/0.01 mmHg). The cyclic pentapeptide sublimed at 230 °C, but tended to decompose at this temperature. The acetone solution, which contained further cyclic pentapeptide, was evaporated, the residue dissolved in chloroform (2 ml) and passed through a small silica gel column. Yield of cyclic compound: Cyclo-D-alanyl-L-alanyltrisarcosyl (38 %), TLC, R_F 0.45 (D), m.p. 270 °C (subl.), *m/e* 355, $[\alpha]_D^{22}$ +14° (*c* 1, MeOH).

Cyclo-di-L-alanyltrisarcosyl

Z-Sar$_2$-L-Ala$_2$-O^{t}Bu. Z-L-Ala$_2$-O^{t}Bu [39] (12 g = 34.5 mmol) (prepared from Z-L-Ala-OH and H-L-Ala – O^{t}Bu [40] using DCC) was hydrogenated and the oil of H-L-Ala$_2$ – O^{t}Bu (7 g = 32.4 mmol) reacted with Z-Sar$_2$-OH [6] (9.5 g = 32.3 mmol) and DCC (7.0 g = 34 mmol) in CH_2Cl_2 (300 ml) resulted in a solid (13 g = 81 %), TLC, R_F 0.6 (C), purified m.p. 90 °C, $[\alpha]_D^{22}$ – 52° (*c* 2, MeOH). (Found: C 58.56; H 7.32; N 11.58. $C_{24}H_{36}N_4O_7$ requires C 58.52; H 7.37; N 11.38).

Z-Sar$_3$-L-Ala$_2$-O^{t}Bu. Z-Sar-OH [41] (1.8 g = 8.1 mmol), H-Sar$_2$-L-Ala$_2$-O^{t}Bu (2.9 g = 8.1 mmol) [obtained by hydrogenation of *Z*-Sar$_2$-L-Ala$_2$-O^{t}Bu (4 g = 8.15 mmol)] and DCC (1.8 g = 8.7 mmol) in CH_2Cl_2 (50 ml) gave a white solid (4 g = 88 %), TLC, R_F 0.35 (C). An analytical sample softened at 70 °C, $[\alpha]_D^{22}$ – 47.6° (*c* 2, MeOH). (Found: C 57.39; H 7.32; N 12.46. $C_{27}H_{41}N_5O_8$ requires C 57.53; H 7.33; N 12.43).

Z-Sar$_3$-L-Ala$_2$-OH. Z-Sar$_3$-L-Ala$_2$O^{t}Bu (3.6 g = 6.4 mmol) was dissolved in ice-cooled trifluoroacetic acid (20 ml), stirred at room temperature for 40 min, evaporated and poured into ether. The ether was decanted, the solid dissolved in acetone and precipitated with ether (2.6 g = 80 %), TLC, R_F 0.4 (G), softened at 85 °C. (Found: C 54.65; H 6.53. $C_{23}H_{33}N_5O_8$ requires C 54.43; H 6.55).

Z-Sar$_3$-L-Ala$_2$-OTcp. Z-Sar$_3$-L-Ala$_2$-OH (1.7 g = 3.35 mmol), HOTcp (1.2 g = 6.1 mmol) and DCC (0.85 g = 4.1 mmol) resulted in a semi-solid (2 g = 87 %), TLC, R_F 0.55 (C). A sample for analysis was crystallised from acetone – ether, softened at 80 °C. (Found: C 50.97; H 5.52; N 10.16. $C_{29}H_{34}N_5O_8Cl_3$ requires C 50.70; H 4.99; N 10.19).

Cyclisation. Z-Sar$_3$-L-Ala$_2$-OTcp (1.9 g = 2.77 mmol) was hydrogenated and the HCl.H-Sar$_3$-L-Ala$_2$-OTcp (1.5 g = 25.6 mmol = 92 %) cyclised. The residue after passage through an ion-exchange column was taken into water to remove undissolved DC-urea and the evaporated residue which showed one main spot on TLC belonging to a cyclic pentapeptide and one minor spot which presumably belongs to the corresponding cyclic decapeptide was chromatographed on a silica gel column. Yield of cyclic compound: Cyclo-di-L-alanyltrisarcosyl (35.4 %), TLC, R_F 0.45 (D), m.p. 225 – 230 °C, *m/e* 355, $[\alpha]_D^{22}$ +20° (*c* 1, MeOH).

Cyclo-L-alanylsarcosyl-D-alanyldisarcosyl

Z-L-AlaSar-D-Ala-OMe. D-Alanine methyl ester hydrochloride (5.8 g = 41.7 mmol), Et_3N (4.3 g = 42.5 mmol), Z-L-AlaSar-OH (12 g = 41 mmol) and DCC (9 g = 43.5 mmol) gave a viscous oil (13 g = 84 %), TLC, R_F 0.6 (C). A sample was purified. (Found: C 57.15; H 6.64; N 10.99. $C_{18}H_{25}N_3O_6$ requires C 56.98; H 6.64; N 11.08).

Z-Sar$_2$-L-AlaSar-D-Ala-OMe. Z-Sar$_2$-OH (5 g = 17 mmol), H-L-AlaSar-D-Ala-OMe (4.2 g = 17.1 mmol) [obtained by hydrogenation of Z-L-AlaSar-D-Ala-OMe (6.5 g = 17.2 mmol)], DCC (3.9 g = 18.9 mmol) gave a foamy solid (7.2 g = 81 %) which on further purification was homogeneous by TLC, R_F 0.4 (C) softened at 65 °C, $[\alpha]_D^{22}$ +5.6° (*c* 2, MeOH).

Z-Sar$_2$-L-AlaSar-D-Ala-OH. Z-Sar$_2$-L-AlaSar-D-Ala-OMe (7 g = 13.4 mmol) and NaOH (14

ml) gave a solid (6.1 g = 90 %) TLC, R_F 0.5 (G), softened at 70 °C, $[\alpha]_D^{22}$ − 5.05° (*c* 2, MeOH). (Found: C 54.36; H 6.69; N 13.96. $C_{23}H_{33}N_5O_8$ requires C 54.43; H 6.55; N 13.80).

Z-Sar$_2$-L-AlaSar-D-Ala-OTcp. Z-Sar$_2$-L-AlaSar-D-Ala-OH (4 g = 7.9 mmol), HOTcp (2.3 g = 11.7 mmol) and DCC (1.8 g = 8.7 mmol) gave a solid (5.1 g = 94 %), TLC, R_F 0.45 (C), crystallised from acetone – ether, softened at 80 °C. (Found: C 51.10; H 5.12. $C_{29}H_{34}H_5O_8Cl_3$ requires C 50.70; H 4.99).

Cyclisation. Z-Sar$_2$-L-AlaSar-D-Ala-OTcp (1 g = 1.46 mmol) was hydrogenated and the HCl.H-Sar$_2$-L-AlaSar-D-Ala-OTcp (0.8 g = 1.36 mmol = 94 %) cyclised. After evaporation of the pyridine the residue was taken into methanol (5 ml) and undissolved material filtered off. This was pure cyclo-L-alanylsarcosyl-D-alanylsarcosyl. The solution was kept at 0 °C overnight and filtered. The precipitate now consisted of some DC-urea and a cyclic tetrapeptide different from that isolated above, and assigned to cyclo-L-alanylsarcosyl-L-alanylsarcosyl (see synthesis). The filtrate was diluted with methanol (100 ml) and passed through an ion-exchange column. The remaining solid after removal of methanol was dissolved in acetone (5 ml) and kept at 0 °C overnight. The precipitate, which showed one spot on TLC (D), melted sharply at 224 °C and had *m/e* = 355 which is the required molecular weight for the cyclic pentapeptide. The filtrate contained more of this cyclic pentapeptide plus two other compounds which were separated by column chromatography on silica gel. The first eluted compound was cyclo-disarcosyl, and the third was the cyclic pentapeptide described above. The second eluted compound, the major product, showed *m/e* = 355 and a fragmentation pattern identical to that of the cyclic pentapeptide isolated above. The latter melted over a wide range, but became more crystalline when dissolved in acetone (2 ml), allowing the solvent to evaporate by leaving the solution open to the air and finally washing with a small amount of acetone. The dried product melted sharply at 274 °C. Yields of cyclic compounds: The major cyclic pentapeptide is suggested to be: Cyclo-L-alanylsarcosyl-D-alanyldisarcosyl (11.3 %). TLC R_F 0.45 (D), m.p. 274 °C, *m/e* 355, $[\alpha]_D^{22}$ − 7° (*c* 1, MeOH) and the minor cyclo-L-alanylsarcosyl-L-alanyldisarcosyl (6.2 %), TLC R_F 0.5 (D), m.p. 224 °C, *m/e* 355, $[\alpha]_D^{22}$ 0° (*c* 0.5, MeOH). The NMR spectra are identical. Further were isolated cyclo-L-alanylsarcosyl-D-alanylsarcosyl (11 %) sublim. temp. 290 °C/0.01 mmHg, m.p. > 350 °C, *m/e* 284, cyclo-L-alanylsarcosyl-L-alanylsarcosyl (2.7 %), sublim. temp. 220 °C/0.01 mmHg, m.p. 340 °C, *m/e* 284 and cyclo-disarcosyl (10.3 %).

Cyclo-L-alanyl-D-alanyl-L-alanyldisarcosyl

Z-L-Ala-D-Ala-L-AlaSar$_2$-OMe. Z-L-Ala-D-Ala-OH (6.2 g = 21.1 mmol) (prepared from Z-L-Ala-OH and HCl.H-D-Ala-OMe using DCC, the protected dipeptide methyl ester was hydrolysed), H-L-AlaSar$_2$-OMe (5.8 g = 23.6 mmol (obtained by hydrogenation of Z-L-AlaSar$_2$-OMe (9.3 g = 24.6 mmol)) and DCC (4.8 g = 23.3 mmHg) in CH_2Cl_2 (70 ml) resulted in a foamy solid (9 g = 82 %), TLC, R_F 0.45 (C). An analytical sample softened at 80 °C, $[\alpha]_D^{22}$ + 3.2° (*c* 2, MeOH). (Found: C 55.25; H 6.66; N 13.38. $C_{24}H_{35}N_5O_8$ requires C 55.26; H 6.76; N 13.43).

Z-L-Ala-D-Ala-L-AlaSar$_2$OH. Z-L-Ala-D-Ala-L-AlaSar$_2$-OMe (7.2 g = 13.8 mmol) in NaOH (15 ml) (2 h) gave a solid which was crystallised from acetone – ether (6.5 g = 93 %), TLC, R_F 0.4 (G), softened at 85 °C, $[\alpha]_D^{22}$ − 1° (*c* 2, MeOH). (Found: C 54.60; H 6.65; N 13.92. $C_{23}H_{33}N_5O_8$ requires C 54.43; H 6.55; N 13.80).

Z-L-Ala-D-Ala-L-AlaSar$_2$-OTcp. Z-L-Ala-D-Ala-L-AlaSar$_2$-OH (3 g = 5.9 mmol), HOTcp (1.7 g = 8.6 mmol) and DCC (1.35 g = 6.55 mmol) resulted in a white powder (3.6 g = 89 %), TLC, R_F 0.4 (C), softened at 85 °C.

Cyclisation. Z-L-Ala-D-Ala-L-AlaSar$_2$-OTcp (0.9 g = 1.3 mmol) was hydrogenated and the HCl.H-L-Ala-D-Ala-L-AlaSar$_2$-OTcp (0.7 g = 1.2 mmol = 91 %) cyclised. The residue after passage through an ion-exchange column was dissolved in acetone (5 ml) and kept overnight at 0 °C. The precipitate contained only a few mg of the cyclic pentamer and some DC-urea which was removed by sublimation, to 200 °C at reduced pressure. The filtrate was evaporated and purified on a silica gel column to give a white compound homogeneous by TLC, but which melted over a wide range. The solid became crystalline when dissolved in acetone (2 ml) and allowed to evaporate to dryness by keeping the sample open in a refrigerator. The residue, cyclo-L-alanyl-D-alanyl-L-alanyldisarcosyl, was washed carefully with acetone and the white powder isolated (28 %) possessed a relatively sharp melting point, m.p. 208 – 210 °C, TLC R_F 0.6 (D), *m/e* 355, $[\alpha]_D^{22}$ = 0° (*c* 2, MeOH).

Cyclo-D-alanyl-L-alanylsarcosyl-D-alanylsarcosyl

Z-Sar-D-Ala-L-AlaSar-D-Ala-OMe. Z-Sar-D-Ala-OH (5 g = 17 mmol), H-L-AlaSar-D-Ala-OMe (4.2 g = 17.1 mmol) [obtained by hydrogenation of Z-L-AlaSar-D-Ala-OMe (6.5 g = 17.2 mmol)] and DCC (4 g = 19.4 mmol) resulted in a foamy solid (7 g = 79 %), TLC, R_F 0.45 (C). An analytical sample softened at 50 °C, $[\alpha]_D^{22}$ + 39.6° (*c* 2, MeOH). (Found: C 54.92; H 6.91; N 13.68. $C_{24}H_{35}N_5O_8$ requires C 55.26; H 6.76; N 13.43).

Z-Sar-D-Ala-L-AlaSar-D-Ala-OH. Z-Sar-D-Ala-L-AlaSar-D-Ala-OMe (7.3 g = 14 mmol) in NaOH (13 ml) (3 h) gave a solid, recrystallised from acetone – ether (6.6 g = 93 %), TLC, R_F 0.5 (G), m.p. 86 °C, $[\alpha]_D^{22}$ + 36.7° (*c* 2, MeOH). (Found: C 54.54; H 6.50. $C_{23}H_{33}N_5O_8$ requires C 54.43; H 6.55).

Z-Sar-D-Ala-L-AlaSar-D-Ala-OTcp. Z-Sar-D-Ala-L-AlaSar-D-Ala-OH (4 g = 7.9 mmol), HOTcp (2.3 g = 11.7 mmol) and DCC (1.8 g = 8.7 mmol) gave a solid (4.6 g = 85 %), TLC, R_F 0.7 (C), softened at 75 °C.

Cyclisation. Z-Sar-D-Ala-L-AlaSar-D-Ala-OTcp (1.4 g = 2 mmol) was hydrogenated and the HCl.H-Sar-D-Ala-L-AlaSar-D-Ala-OTcp (1.1 g = 1.9 mmol = 92 %) cyclised. The powder left after passage through an ion-exchange column consisted of two compounds (I and II) with approximately the same R_F-values (TLC, solvent D). Most of the major compound (I) could be filtered off after suspending the powder in acetone. The filtrate which still contained both compounds was passed through a silica gel column and the isolated compounds (I and II) both had *m/e* 355 which is the molecular ion required for the cyclic pentapeptide. The major compound (I) is assumed to be cyclo-D-alanyl-L-alanylsarcosyl-D-alanylsarcosyl (53 %), TLC, R_F 0.6 (D), m.p. 317 °C (sublim.), *m/e* 355, $[\alpha]_D^{22}$ + 18° (*c* 2, MeOH). (Found: C 50.85; H 6.92; N 19.87. $C_{15}H_{25}N_5O_5$ requires C 50.69; H 7.09; N 19.71). The minor compound (II) is assumed to be cyclo-D-alanyl-L-alanylsarcosyl-L-alanylsarcosyl (3 %), TLC, R_F 0.55 (D), m.p. 260 °C, *m/e* 355, $[\alpha]_D^{22}$ + 10° (*c* 0.4, MeOH).

Cyclo-glycyltetrasarcosyl

Z-GlySar-OMe. Sarcosine methyl ester hydrochloride (14 g = 100 mmol), Et_3N (10.5 g = 104 mmol), benzyloxycarbonylglycine [37] (21 g = 100 mmol) and DCC (22 g = 107 mmol) resulted in a viscous oil (25 g = 85 %), TLC, R_F 0.7 (B). A sample was purified. (Found: C 56.92; H 5.91; N 9.60. $C_{14}H_{18}N_2O_5$ requires C 57.13; H 6.17; N 9.52).

Z-GlySar-OH. Z-GlySar-OMe (27 g = 92 mmol) in NaOH (93 ml) (2½ h) gave a solid, recrystallised from acetone (20 g = 78 %), TLC, R_F 0.5 (F), m.p. 95 – 97 °C. (Found: C 55.26; H 5.62; N 10.01. $C_{13}H_{16}N_2O_5$ requires C 55.71; H 5.75; N 10.0).

Z-GlySar$_4$-OMe. Z-GlySar-OH (6.5 g = 23.2 mmol), H-Sar$_3$-OMe [6] (5.7 g = 23.2 mmol) and DCC (5.2 = 25.2 mmol) gave a foamy solid (9.2 g = 78 %), TLC, R_F 0.3 (C), softened at 70 °C.

Z-GlySar$_4$-OH. Z-GlySar$_4$-OMe (7.5 g = 14.8 mmol) in NaOH (16 ml) (3 h) gave a solid (6.2 g = 85 %), TLC, R_F 0.6 (H), softened at 80 °C. (Found: C 53.5; H 6.36; N 14.19. $C_{22}H_{31}N_5O_8$ requires C 53.54; H 6.33; N 14.19).

Z-GlySar$_4$-OTcp. Z-GlySar$_4$-OH (3 g = 6.1 mmol), HOTcp (2 g = 10 mmol) and DCC (1.5 g = 7.3 mmol) gave a solid, recrystallised from acetone (3.8 g = 90 %), TLC, R_F 0.65 (D), m.p. 135 °C. (Found: C 50.04; H 4.90; N 10.25. $C_{28}H_{32}N_5O_8Cl_3$ requires C 49.98; H 4.79; N 10.41).

Cyclisation. Z-GlySar$_4$-OTcp (2 g = 2.98 mmol) was hydrogenated and the HCl.H-GlySar$_4$-OTcp (1.6 g = 2.8 mmol = 93 %) cyclised. The residue after passage through an ion-exchange column was suspended in water to remove the DC-urea by filtration, the water was removed from the filtrate and acetone (5 ml) added, some insoluble material was filtered off. This was pure cyclo-glycyltetrasarcosyl and a further crop crystallised out when the solution was kept in a refrigerator. A small amount of cyclo-glycyltetrasarcosyl-glycyltetrasarcosyl (TLC) was present in the filtrate but could not be isolated by these methods. Yield of cyclic compound: cyclo-glycyltetrasarcosyl (52.2 %), TLC, R_F 0.25 (D), R_F 0.3 (E), m.p. 230 °C, *m/e* 341.

Cyclo-diglycyltrisarcosyl

Z-GlySar$_2$-OMe. HCl.H-Sar-OMe (7 g = 50 mmol), Et_3N (5.5 g = 54 mmol), Z-GlySar-OH (14 g = 50 mmol) and DCC (11 g = 53 mmol) resulted in an oil (14 g = 77 %), TLC, R_F 0.7 (C) which did not crystallise from acetone on addition of ether. A sample was purified. (Found: C 55.84; H 6.39; N 11.42. $C_{17}H_{23}N_3O_6$ requires C 55.88; H 6.35; N 11.50).

Z-Gly$_2$Sar$_2$-OMe. Z-GlySar$_2$-OMe (10 g = 27.4 mmol) was hydrogenated and the oil of H-GlySar$_2$-OMe (6.3 g = 27 mmol) dissolved in CH_2Cl_2 (200 ml) together with Z-Gly-OH (5.6 g = 27 mmol) and DCC (6.2 g = 30 mmol) and the resulting solid (9 g = 82 %) recrystallised from acetone, TLC, R_F 0.45 (C), m.p. 120 °C. (Found: C 54.81; H 6.59; N 13.11. $C_{20}H_{28}N_4O_7$ requires C 55.03; H 6.47; N 12.84).

Z-SarGly$_2$Sar$_2$-OMe. Z-Sar-OH (4.2 g = 18.8 mmol), H-Gly$_2$Sar$_2$-OMe (5.4 g = 18.8 mmol) [obtained by hydrogenation of Z-Gly$_2$Sar$_2$-OMe (8 g = 19 mmol)] and DCC (4.3 g = 20.8 mmol) gave a solid (7.9 g = 85 %), TLC, R_F 0.6 (D). An analytical sample softened at 65 °C. (Found: C 53.46; H 6.42; N 13.92. $C_{22}H_{31}N_5O_8$ requires C 53.54; H 6.33; N 14.19).

Z-SarGly$_2$-Sar$_2$-OH. Z-SarGly$_2$Sar$_2$-OMe (7.2 g = 14.6 mmol) was hydrolysed for 2 h in NaOH (16 ml). Extraction of the acidified aqueous layer with chloroform afforded only 4.2 g of the corresponding acid. In order to isolate more of the acid, the aqueous layer was evaporated nearly to dryness, and extraction with chloroform gave a further 1.8 g (6 g = 86 %), TLC, R_F 0.7 (H), softened at 90 °C.

Z-SarGly$_2$Sar$_2$-OTcp. Z-SarGly$_2$Sar$_2$-OH (2 g = 4.18 mmol), HOTcp (1.3 g = 6.6 mmol) and DCC (1 g = 4.86 mmol) gave a solid, recrystallised from acetone (2.4 g = 87 %), TLC, R_F 0.7 (D), softened at 90 °C. (Found: C 49.27;

H 4.69. $C_{27}H_{30}N_5O_8Cl_3$ requires C 49.22; H 4.59).

Cyclisation. Z-SarGly$_2$Sar$_2$-OTcp (1.5 g = 2.28 mmol) was hydrogenated and the HCl.H-SarGly$_2$Sar$_2$-OTcp (1.2 g = 2.15 mmol = 94 %) cyclised. The residue after passage through an ion-exchange column was dissolved in methanol (1 ml) and the solvent allowed to evaporate by keeping the sample open in a refrigerator, the residue was washed carefully with methanol to give a white powder of cyclo-diglycyltrisarcosyl (5.7 %), TLC, R_F 0.35 (E), m.p. 209–211 °C, *m/e* 327.

Cyclo-glycylsarcosylglycyldisarcosyl

Z-GlySarGlySar$_2$-OMe. Z-GlySar-OH (5 g = 17.8 mmol), H-GlySar$_2$-OMe (4 g = 17.3 mmol) and DCC (4 g = 19.4 mmol) gave a foamy solid (7.4 g = 87 %), TLC, R_F 0.3 (C). An analytical sample softened at 65 °C. (Found: C 53.42; H 6.36; N 13.91. $C_{22}H_{31}N_5O_8$ requires C 53.54; H 6.33; N 14.19).

Z-GlySarGlySar$_2$-OH. Z-GlySarGlySar$_2$-OMe (6.4 g = 13 mmol) in NaOH (14 ml) (2½ h) gave the acid (5.5 g = 88 %), TLC, R_F 0.65 (H), softened at 80 °C. (Found: C 52.46; H 5.86. $C_{21}H_{29}N_5O_8$ requires C 52.60; H 6.10).

Z-GlySarGlySar$_2$-OTcp. Z-GlySarGlySar$_2$-OH (3 g = 6.3 mmol), HOTcp (1.8 g = 9.1 mmol) and DCC (1.5 g = 7.28 mmol) gave a solid, crystallised from acetone; (3.7 g = 90 %), TLC, R_F 0.65 (D), m.p. 145–147 °C. (Found: C 49.07; H 4.68. $C_{27}H_{30}N_5O_8Cl_3$ requires C 49.22; H 4.59).

Cyclisation. Z-GlySarGlySar$_2$-OTcp (1.35 g = 2.05 mmol) was hydrogenated and the HCl.H-GlySarGlySar$_2$-OTcp (0.9 g = 1.6 mmol = 79 %) cyclised. The residue after passage through an ion-exchange column was chromatographed on a silica gel column and two cyclic compounds were isolated, cyclo-glycylsarcosyl (8.3 %) and cyclo-glycylsarcosylglycyldisarcosyl, the latter melting over a wide range. The cyclic pentapeptide became more crystalline by dissolving in methanol (1 ml) and allowing the solvent to evaporate by keeping the sample open in a refrigerator. The residue was washed carefully with methanol and the white powder (4 %) of cyclo-glycylsarcosylglycyldisarcosyl was homogeneous by TLC, R_F 0.3 (E), had *m/e* 327 and melted at 222–230 °C.

Acknowledgement. I thank Professor J. Dale who contributed to this work through many helpful discussions especially regarding the conformational aspects. This work was supported by the Norwegian Research Council for Science and Humanities and by Borregaard Industries Limited, Sarpsborg.

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Received March 15, 1976.