Amino-acids and Peptides. Part XVIII.[1] Studies Relating to the Synthesis and Conformation of a Novel Bicyclic Peptide System

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The synthesis of a bicyclic system (I) derived by coupling the two fourteen-membered cyclic peptides cyclo-[L-(α-amino)-β-alanylglycyl-D-(α-tosylamino)-β-alanylglycyl] (II) and cyclo-[D-β-aspartylglycyl-β-alanylglycyl] (III), and related bicyclic compounds, is described. The conformations of the compounds are discussed in the light of evidence from ^{1}H n.m.r. spectroscopy and a crystal structure analysis of one of the monocyclic compounds.

INVESTIGATIONS[2] of the molecular structures of the serine proteinases α-chymotrypsin, porcine elastase, trypsin, and subtilysin indicate that they have in common an arrangement of serine, histidine, and aspartic acid residues at the active centre. Although other features determine the specific binding of substrates, an appropriate array of these three residues is essential for the cleavage of the peptide bonds of substrates by these enzymes; the ion-transfer mechanism, which has been proposed[3] for the hydrolysis, requires neighbouring group participation involving all three residues.

This study is concerned with the design of a novel system containing histidine, serine, and aspartic acid residues in a similar relationship in space to that found in the serine proteinases. This system is based on appropriate fourteen-membered cyclotetrapeptides. Earlier studies by Dale,[4] Dunitz,[5] and others, using simpler compounds, suggested that such a ring would be well suited to our purpose. In the hydrocarbon series, cyclotetradecane is the most strain-free cycloalkane after cyclohexane;[6] both assume conformations corresponding to the 'diamond lattice.' The cyclotetradecane molecule (IV) is compact and centrosymmetrical, and has a diameter similar to that of the α-helix of polypeptides.[7] In the light of the influence of functions (CO, NH, *etc.*) in the ring, we deduced that the most likely conformation for a fourteen-membered cyclotetrapeptide derived from alternating α- and β-amino-acids, would be represented by (V) for an unsubstituted

[1] Part XVII, B. K. Handa and C. H. Hassall, preceding paper.

[2] *Inter alia*, B. W. Matthews, P. B. Sigler, R. Henderson, and D. M. Blow, *Nature*, 1967, **214**, 652; D. M. Shotton and H. C. Watson, *Phil. Trans.*, 1970, **B257**, 111; H. Diesenhofer and W. Streigemann, 'Stockholm Symposium on the Structure of Biological Molecules,' 1973, p. 98; C. S. Wright, *J. Mol. Biol.* 1972, **67**, 151.

[3] A. Liljas and M. G. Rossmann, *Ann. Rev. Biochem.*, 1974, **43**, 498.

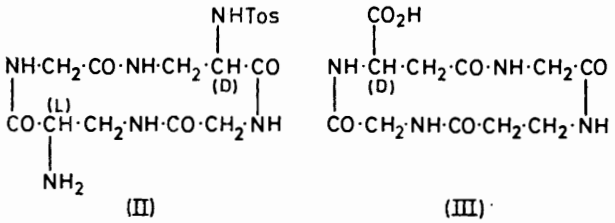
[4] J. Dale, *J. Chem. Soc.*, 1963, 93.

[5] J. D. Dunitz and E. F. Meyer, *Helv. Chim. Acta*, 1965, **48**, 1441.

[6] J. Sicher, *Progr. Stereochem.*, 1962, **3**, 204.

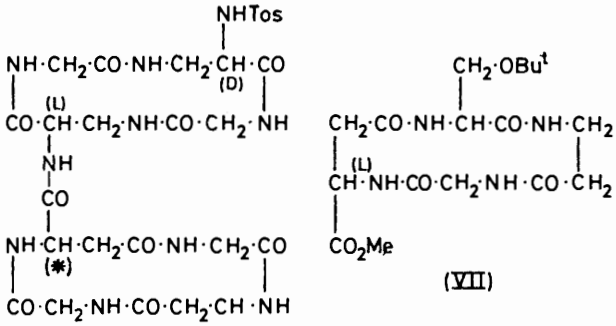
[7] C. H. Hassall, in 'Chemistry and Biology of Peptides: Proceedings of the Third American Peptide Symposium,' ed. J. Meienhofer, Ann Arbor Science, Ann Arbor, 1972, p. 153.

ring, and (VI) for a ring with substitution as in compound (III). This followed from the preference for sp^2-hybridised carbon atoms to be disposed externally, for *trans*-coplanar rather than *cis*-amide bonds, and for substitution to be adjacent to a corner carbon atom, since the two atoms adjacent to the corner atom are joined by *gauche*-bonds and carbon–carbon single bonds



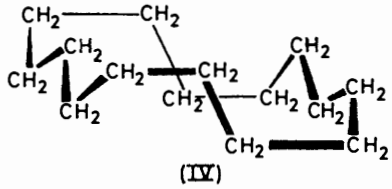
(II)

(III)



(I) * = D (IX) * = L

(VII)



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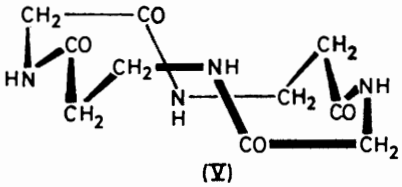

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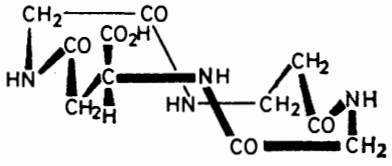
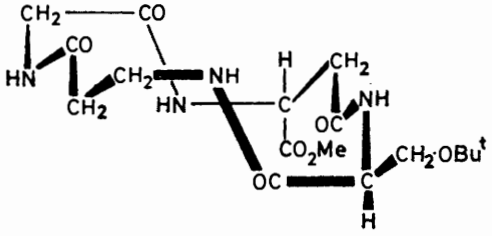


(VI)



(VIII)

graphic investigation by direct methods. This established [12] that cyclo[-L-Ser(Bu^t)-β-Ala-Gly-D-Asp-OMe] (VII) has the ring conformation (VIII).

If such a conformation is assumed for a single ring, it is possible to design a linkage between two rings that favours the formation of a cylindrical structure. This is represented in Figure 1, in which the amide group linking the rings is attached to separate carbon atoms in adjacent positions for each ring, of opposite configuration and each with a pseudoaxial relationship to the ring. This has the effect of orienting NH functions of one ring towards carbonyl functions of the other, to allow intramolecular hydrogen bonding. Such a 'stacking' of single molecules of cyclic peptides occurs [12] in the crystal of compound (VII) as shown (Figure 3) but, in the absence of an interannular covalent linkage, only two

next to most groups can take up *gauche*- as easily as *trans*-bonds. From these considerations [8,9] and from Celmer's treatment [10] as applied to macrolide ring systems, it was predicted that two conformations out of fourteen possible modifications were favoured for fourteen-membered cyclodepsipeptides related to serratamolide,[11] and the conformation (V) was favoured for the corresponding cyclotetrapeptide.[7] Some support for the conformation (V) has come from ^{1}H n.m.r. studies but, more certainly, from a recent *X*-ray crystallo-

[8] J. Dale and R. Coulan, *J. Chem. Soc.*, 1964, 182.
[9] J. Dale, *Angew. Chem. Internat. Edn.*, 1966, **5**, 1000.
[10] W. D. Celmer, in 'Biogenesis of Antibiotic Substances,' Czechoslovak Academy of Sciences, Prague, 1965, p. 99.
[11] C. H. Hassall, T. G. Martin, J. A. Schofield, and J. O. Thomas, *J. Chem. Soc.* (*C*), 1967, 997.
[12] I. L. Karle, B. K. Handa, and C. H. Hassall, *Acta Cryst.*, 1975, **B31**, 555.

hydrogen bonds are formed between the two molecules. The presence of the methoxycarbonyl group causes a lateral displacement of adjacent rings to produce a 'skewed cylinder' in this case. The evidence of the conformation (VIII) of the cyclic peptide (VII) and the construction of models of the bicyclic compound (I) lead us to believe that a cylindrical arrangement with four hydrogen bonds will be favoured and will take the form shown in Figure 2. The representation Figure 2b indicates the manner in which the seryl side chain in the cyclopeptide (VIII) is disposed in a pseudoequatorial relationship to the ring. In the same way, histidyl and aspartyl side chains of residues in separate rings of such a cylindrical system may be expected to assume similar

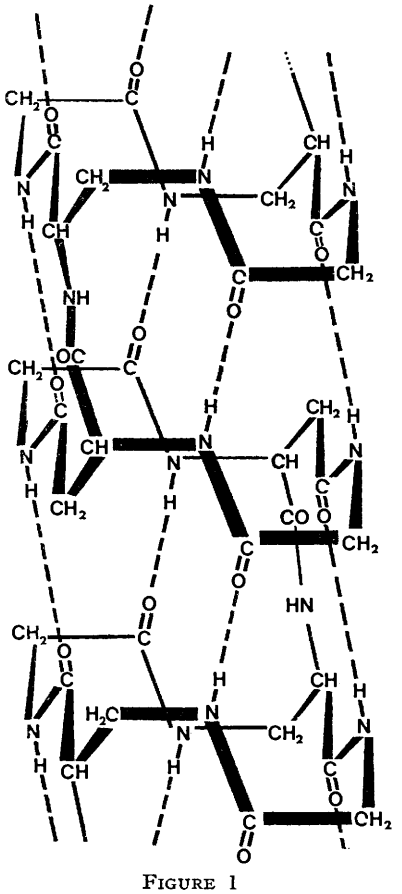


FIGURE 1

equatorial relationships. This is the basis for design of a structure with these three residues in a spatial relationship simulating that in a proteinase. Related intermolecular stacking of peptides has been observed in several cases.[13,14]

We have synthesised the bicyclic compound cyclo-[D-β-aspartylglycyl-β-alanylglycyl]–cyclo[L-(α-amino)-β-alanylglycyl-D-(α-tosylamino)-β-alanylglycyl] (I) in 30% yield by coupling the azide of the cyclic peptide (III) with the aminocyclopeptide (II). Analysis of the product both by combustion and by determination of the amino-acid composition of the hydrolysate confirmed the identity of the bicyclic product. It was amorphous; moreover, it has not yet proved possible to

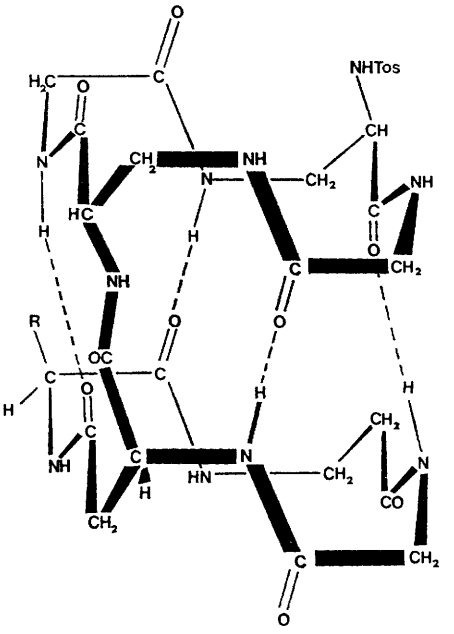


FIGURE 2 a; R = $CH_2 \cdot OBu^t$ b; R = H

prepare crystalline derivatives suitable for *X*-ray crystallographic analysis. The related compounds (IX)—(XI) have been prepared by coupling the appropriate cyclic tetrapeptides in a similar manner. They are all amorphous, and not very soluble.

Following the determination of the conformation of the cyclotetrapeptide (VII) in the crystal, a ^1H n.m.r. study was undertaken to define the conformation in

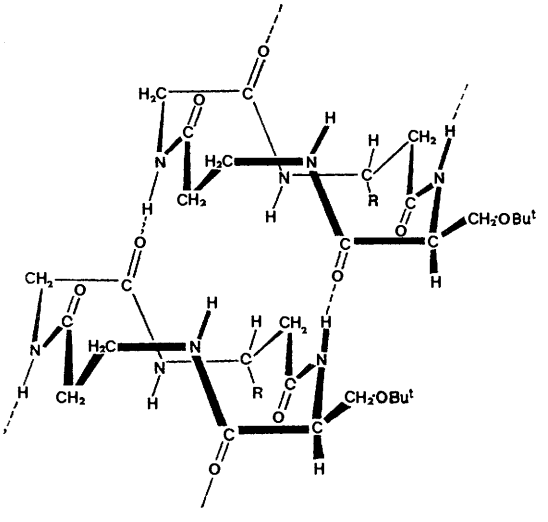


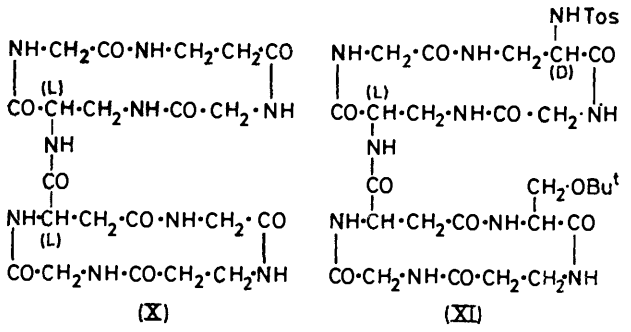
FIGURE 3 R = CO_2Me

solution. Initially, the temperature dependence and rates of exchange of the four NH protons were examined.

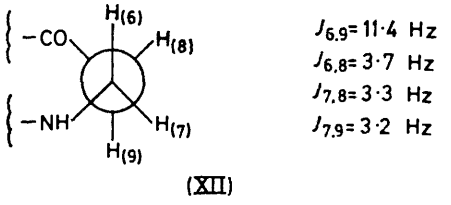
[13] M. Dobler, J. D. Dunitz, and J. Krajewski, *J. Mol. Biol.*, 1969, **42**, 603.

[14] Yu. A. Ovchinnikov and V. T. Ivanov, *Tetrahedron*, 1975, **31**, 2177, and references therein.

The results indicated that the behaviour of the NH protons of the aspartyl and β-alanyl residues was less temperature dependent and that these protons were

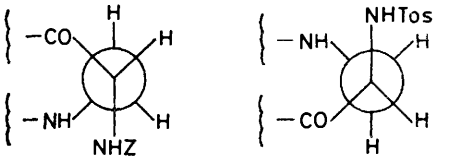


(X) (XI)



$J_{6,9}$ = 11·4 Hz
$J_{6,8}$ = 3·7 Hz
$J_{7,8}$ = 3·3 Hz
$J_{7,9}$ = 3·2 Hz

(XII)



(XIII)

less rapidly exchanged for deuterium [in $(CD_3)_2SO$–D_2O] than the other pair. In the crystal, it is the glycyl and seryl NH protons which form intermolecular hydrogen bonds, but these are unlikely to be present in solution at ambient temperature. The observed differences in temperature gradient and exchange rate for these protons in this monocyclic peptide, in solution, could be attributed to intermolecular hydrogen bonding effects, but we consider it more likely that they are due to shielding of two of the protons from the solvent by steric effects.

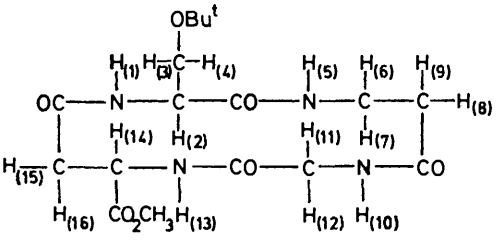
Analysis of part of the n.m.r. spectrum of (VII) in a variety of solvents demonstrated the relative invariance of the vicinal and geminal coupling constants around the ring. Even in hexafluoroacetone 1.5-deuterium oxide, a solvent known to break intra- and inter-molecular hydrogen bonds, the peptide backbone appears to assume the same rigid conformation.

Chemical shifts and coupling constants for (VII) are recorded in Table 1. In the corresponding cyclic peptide (III) in which serine is replaced by glycine, the $^3J_{\alpha\beta}$ values for the aspartyl residue are 7.5 and 0.0 Hz (hexafluoroacetone solvent), in contrast to the corresponding values (5.0 and 2.5 Hz) for (VII). This suggests that there is a slight modification in the conformation of this section of the fourteen-membered ring, with the C_α–C_β torsion angle μ_4 changing by *ca.* 20°. However, the remaining peptide backbone conformation seems little changed from that in (VIII); the small change in μ_4 is evidently due to the steric requirement of the bulky CH_2·OBu^t substituent.

A comparison of the torsion angles found in the crystal conformation (VIII) and in solution is made in Table 2. Allowing for ±10° in application of the $\cos^2\phi$ relationship, the agreement between crystal and solution seems reasonable. The differences suggest that in solution the conformation of the peptide (VII) is such that two of the amide bonds are more tilted from the average plane through the peptide backbone than in the crystal.

TABLE 1

Chemical shifts and coupling constants for the cyclotetrapeptide



δ(p.p.m. from internal Me_4Si ± 0.01)

Solvent	H(1)	H(2)	H(3)	H(4)	H(5)	H(6)	H(7)	H(8)	H(9)	H(10)	H(11)	H(12)	H(13)	H(14)	H(15)	H(16)
$(CD_3)_2SO$	8.18	4.06	3.48	3.48	7.08	*	*	2.32	2.32	8.64	3.73	3.54	7.03	4.73	2.95	2.64
CD_3OD		4.18	*	*		*	*	2.46	2.46		3.93	3.66		4.84	3.16	2.80
C_5D_5N	9.52	4.71	*	*	7.90	*	*	2.45	2.66	9.82	4.24	3.91	7.85	5.19	3.34	3.03
HFA †		4.32	3.77	3.77		*	*	2.62	2.62		4.14	3.78		4.99	3.36	2.95

J/Hz (±0.1)

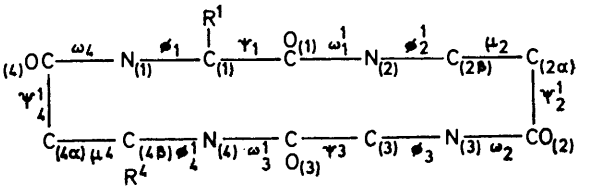
Solvent	$J_{1,2}$	$J_{2,3}+J_{2,4}$	$J_{5,6}+J_{5,7}$	$J_{6,8}$	$J_{6,9}$	$J_{7,8}$	$J_{7,9}$	$J_{8,9}$	$J_{10,11}$	$J_{10,12}$	$J_{11,12}$	$J_{13,14}$	$J_{14,15}$	$J_{14,16}$	$J_{15,16}$
$(CD_3)_2SO$	5.0	10.0	12.0	*	*	*	*	*	6.0	6.0	−15.0	9.5	3.0	4.5	−16.5
CD_3OD		8.5		*	*	*	*	*			−16.5		2.0	4.5	−17.0
C_5D_5N	6.0	9.5	*	3.7	11.4	3.7	3.3	−16.5	5.7	6.0	−16.2	9.8	2.7	4.5	−17.5
HFA		8.0		*	*	*	*	*			−17.0		2.5	5.0	−17.5

* Not determined. † $(CF_3)_2CO$,1.5D_2O.

Confirmation of a rigid conformation for (VII) in solution came from complete analysis of the n.m.r. signals for the CH_2·CH_2 fragment of the β-alanine residue. Treatment as a four-spin system (solvent [$^2H_5$]pyridine) gave three vicinal couplings which can be assigned as shown (XII); the large value (11.4 Hz) for $J_{6,9}$ rules out any averaging process.

TABLE 2

Torsion angles (°) in solution and in the crystal



$R^1 = CH_2 \cdot OBu^t$ $R^4 = CO_2Me$

	Crystal	Solution *		Crystal	Solution *
ϕ_1	−75.2	−90	μ_2	−57.6	−60
ϕ_2^1	−103.4	−90	μ_4	57.0	60
ϕ_3	69.3	30	μ_3	25.5	0
ϕ_4	98.7	115	ψ_4^1	−163.2	−180

* In solution, ±10° is probable average error.

Interpretation of the spectrum of the diamino-peptide (II) in the Z-protected form was difficult owing to complexity and overlapping in the regions of interest. However, at 220 MHz in hexafluoroacetone sesquideuterate, some fine structure could be resolved. Narrow triplets at 4.03 (ΣJ 8.4 Hz) and 4.49 (ΣJ 8.6 Hz) in the 220 MHz spectrum can be assigned to the two C*H*·CH_2 protons, the lower field triplet being attributed to the CH unit adjacent to the tosylamino-group. One of these two methylene groups can also be distinguished as the AB portion of an ABX system, with δ_A 3.48, δ_B 3.39, J_{AB} 15.0 Hz, and $J_{AX} = J_{BX} = 4$ Hz. Finally, one of the glycyl-CH_2 signals is distinguishable as an AB quartet (J_{AB} −15.0 Hz). The narrow triplets are in accord with a *gauche* relationship between the vicinal protons as represented by the partial conformation (X) with the amino-function pseudoaxial in each case. This is as expected for partial conformations (XIII) of the ring similar in conformation to that shown to exist for the related cyclic peptide (VII).

The structure (Figure 2) deduced for the bicyclic compound (I) incorporates two fourteen-membered rings represented in conformations which had not, as yet, been supported by convincing experimental evidence. The new data derived from the *X*-ray investigation [12] and these ^1H n.m.r. studies indicates that, in the monocyclic, fourteen-membered peptides (II) and (VII) the ring conformations are rigid and in accord with those postulated for the bicyclic system (I).

EXPERIMENTAL

M.p.s were determined with a Kofler hot-stage apparatus. I.r. spectra were determined for potassium bromide discs with a Perkin-Elmer 257 spectrophotometer. N.m.r. spectra were determined at 100 MHz with Varian HA-100 and XL-100 spectrometers, and also at 220 MHz with a Varian HR-220 instrument (P.C.M.U., Harwell). Mass spectra were determined with an A.E.I. MS9 double-focusing spectrometer. Amino-acid analyses were performed with a JEOL 6AH autoanalyser.

Cyclo-[L-β-*aspartylglycyl*-β-*alanylglycyl*] *Hydrazide.*—Hydrazine hydrate (99—100%) (100 ml) was added to a suspension of cyclo-[L-β-aspartylglycyl-β-alanylglycyl] (300 mg, 1.59 mmol) prepared as in the case of the corresponding D-isomer [15] in methanol (25 ml). The mixture was shaken vigorously for 15 h and then water (100 ml) was added. Removal of the solvent under high vacuum left an amorphous product. Further treatment with hot water gave the *product* (425 mg, 85%) (Found: N, 25.0. $C_{11}H_{18}N_6O_5$, H_2O requires N, 25.3%), M^+ 314.133 87 ± 15 ($C_{11}H_{18}N_6O_5$ requires *M*, 314.133 86). The i.r. spectrum did not include CO_2Me absorption (1 700—1 750 cm^{-1}).

Cyclo-[L-β-*aspartylglycyl*-β-*alanylglycyl*] *Azide.*—Sodium nitrite (30 mg, 0.43 mmol) in ice-cold water (0.5 ml) was added to a suspension of the foregoing hydrazide (100 mg, 0.30 mmol) in 1.0M-hydrochloric acid (1 ml) at 0 °C, and the mixture was shaken for 15 min. The precipitate obtained after neutralising the excess of acid was washed immediately with ice-cold water (3 ml) and then with acetone (15 ml) and ether (20 ml), to give the *product* (89 mg, 85%) as an amorphous solid (Found: C, 40.8; H, 4.5. $C_{11}H_{15}N_7O_5$ requires C, 40.6; H, 4.6%), $\nu_{max.}$ 2 105 cm^{-1} (CON_3).

Cyclo-[D-β-*aspartylglycyl*-β-*alanylglycyl*]-*Cyclo-*[L-(α-*amino*)-β-*alanylglycyl*-D-(α-*tosylamino*)-β-*alanylglycyl*] (I).—Cyclo-[D-β-aspartylglycyl-β-alanylglycyl] azide (72 mg, 0.15 mmol), synthesised from the corresponding hydrazide [15] as for the foregoing L-isomer, was added to a solution of cyclo-[L-(α-amino)-β-alanylglycyl-D-(α-tosylamino)-β-alanylglycyl] (II) [16] (50 mg, 0.11 mmol) in dry dimethylformamide (4 ml) at 0 °C. The mixture was stirred for 3 h and set aside for 72 h at 0 °C. After filtration, the solution was treated with acetone (15 ml) and ether (20 ml) to yield an amorphous solid. Treatment of the solid with hot water–ethanol (1 : 1 v/v; 3 × 10 ml), acetone (10 ml), and ether (15 ml) yielded the *product* (37 mg, 30%) as an amorphous solid, m.p. >350° (Found: C, 43.6; H, 5.5; N, 18.2. $C_{28}H_{38}N_{10}O_{11}S,3H_2O$ requires C, 43.3; H, 5.7; N, 18.0%), δ[$(CF_3)_2CO$,1.5D_2O] 7.3—7.8 (4 H, AA'BB', $MeC_6H_4 \cdot SO_2$) and 1.0—4.0br (2 H); amino-acid analysis of the hydrolysate: Asp, 0.96; Gly, 4.0; βAla, 1.1; D-α-tosyl-αβ-diaminopropionic acid (Tos-Dip), 0.94.

Cyclo-[L-β-*aspartylglycyl*-β-*alanylglycyl*]-*cyclo-*[L-(α-*amino*)-β-*alanylglycyl*-D-(α-*tosylamino*)-β-*alanylglycyl*] (IX).—The foregoing L-cyclotetrapeptide azide (72 mg, 0.15 mmol) was added as in the previous case to a solution of cyclo-[L-(α-amino)-β-alanylglycyl-D-(α-tosylamino)-β-alanylglycyl] (II) [16] (50 mg, 0.11 mmol) in dry dimethylformamide (4 ml) at 0 °C. The mixture was stirred for 3 h and set aside for 72 h at 0 °C. Work-up as before yielded the *product* (55 mg, 45%) as an amorphous solid, m.p. >350° (Found: C, 45.7; H, 5.3; N, 18.8. $C_{28}H_{38}N_{10}O_{11}S,H_2O$ requires C, 45.4; H, 5.4; N, 18.8%), ^1H n.m.r. spectrum [$(CF_3)_2CO$,1.5D_2O] identical with that of the diastereoisomeric compound (I); amino-acid analysis of the hydrolysate: Asp, 0.89; Gly, 4.00; βAla, 1.08; Tos-Dip, 1.1.

[15] C. H. Hassall, D. G. Sanger, and B. K. Handa, *J. Chem. Soc.* (*C*), 1971, 2814.

[16] Part XVI, C. H. Hassall, R. G. Tyson, and K. K. Chexal, *J.C.S. Perkin I*, 1976, 2010.

Cyclo-[L-(α-*amino*)-β-*alanylglycyl*-β-*alanylglycyl*]–*cyclo*-[L-β-*aspartylglycyl*-β-*alanylglycyl*] (X).—The azide (25 mg, 0.077 mmol) was added to a solution of cyclo-[L-(α-amino)-β-alanylglycyl-β-alanylglycyl] (15 mg, 0.044 mmol) in hexamethylphosphoramide (2 ml) at 0 °C. The mixture was stirred for 8 h, then set aside at 0 °C for 72 h, and filtered. An excess of dry ethyl acetate was added to obtain more precipitate from the filtrate. Normal work-up gave the *product* (6 mg, 20%) as an amorphous solid, m.p. >300°, virtually insoluble in all the common solvents investigated; amino-acid analysis of the hydrolysate: Asp, 1.06; Gly, 4.05; βAla, 1.99, in good accord with the required values for the bicyclic system and well differentiated from the values for the related monocyclic peptides.

Cyclo-[(O-*t-butyl*)-L-*seryl*-β-*alanylglycyl*-L-β-*aspartyl*] *Hydrazide*.— Cyclo-[(*O*-t-butyl)-L-seryl-β-alanylglycyl-(*O*-methyl)-L-β-aspartyl] [1] (0.2 g, 0.5 mmol) was dissolved in purified dimethylformamide (10 ml). Hydrazine hydrate (2 ml) was added, and the mixture set aside at room temperature (24 h). Removal of the solvent at 25 °C left a white solid which was triturated with methanol and filtered off (0.15 g, 75%). Dissolution in the minimum quantity of water–dimethylformamide (1 : 1) followed by concentration under vacuum yielded the *product* (0.12 g, 60%) as a white solid, m.p. 290° (Found: C, 48.0; H, 7.15; N, 20.9. $C_{16}H_{28}N_6O_6$ requires C, 48.0; H, 7.05; N, 21.0%), M^+ 400.

Cyclo-[L-(α-*amino*)-β-*alanylglycyl*-D-(α-*tosylamino*)-β-*alanylglycyl*]–*Cyclo*-[(O-*t-butyl*)-L-*seryl*-β-*alanylglycyl*-L-β-*aspartyl*] (XI).—The foregoing hydrazide (50 mg, 0.15 mmol) was suspended in purified dimethylformamide (1 ml). After cooling to −20 °C, a precooled 0.5M-solution of hydrogen chloride (2 equiv.) in dimethylformamide (0.55 ml) was added, with stirring. The clear solution obtained in this way was treated with a precooled solution of isopentyl nitrite (0.75 ml of a 2% solution in dimethylformamide, 1 equiv.). The mixture was stirred for 15 min at −20 °C and then neutralised with triethylamine (1.3 ml of 2% solution in dimethylformamide, 2 equiv.). Cyclo-[L-(α-amino)-β-alanylglycyl-D-(α-tosylamino)-β-alanylglycyl] (II) (55 mg, 0.12 mmol) in dimethylformamide (4 ml) at −10 °C was added dropwise to the solution. The mixture was stirred at −20 °C (2 h) and then maintained at −4 °C for 3 days. The solid obtained by evaporation was triturated with dimethylformamide–water (1 : 1) at 90 °C, filtered off, and further triturated with boiling water–ethanol (1 : 1) to yield the *product* (50 mg, 50%) (Found: C, 46.3; H, 6.0; N, 16.0. $C_{33}H_{48}O_{12}N_{10}S,2.5H_2O$ requires C, 46.4; H, 6.3; N, 16.4%). The ^1H n.m.r. spectrum [$(CD_3)_2SO$] included the expected Tos and Bu^t resonances. The amino-acid analysis of the hydrolysate gave: Asp, 0.98; Ser, 0.97; Gly, 2.82 (97% recovery).

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