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Synthesis and Physicochemical Properties of Cyclic Peptides[1,2)]

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A series of cyclic peptides varying in ring size and in amino acid constituents was synthesized. Cyclization reactions of linear peptides were carried out by the azide, *p*-nitrophenyl ester, *N*-hydroxysuccinimide ester and diethyl phosphorocyanidate methods. Intermolecular cyclodimerization was also applied for the preparation of a cyclic hexapeptide. The reaction yield in each cyclization method was found to be sufficient for preparative purposes. Conformational analysis of the cyclic peptides was carried out by using proton nuclear magnetic resonance. The temperature dependency of peptide NH signals revealed that hexapeptides with the cyclo(–Gly–Xxx–Gly–)$_2$ sequence are stabilized by intramolecular hydrogen bonding and are resistant to temperature-induced conformational change.

Keywords——cyclic peptide; peptide synthesis; cyclization; conformation; ^{1}H-NMR signal; peptide amide proton; hydrogen bond

Many naturally occurring cyclic peptides have been reported, and some have potent biological activities. For example, it has been shown that a cyclic decapeptide, antamanide, serves as a sodium ionophore by transporting the metal ion in lipid membranes, and this property is due to the capability to include a sodium ion specifically in its macrocyclic cavity.[3)] Synthetic cyclic peptides could be promising models of host molecules in host–guest chemistry, since the conformational flexibility of the peptide backbone is considerably reduced from that of open-chain analogs. Furthermore, design of chiral environments with various functionalities in and around the peptide backbone is possible by choosing appropriate amino acid residues as constituents. It is thus of interest to prepare a series of cyclic peptides with various amino acid constituents and various ring sizes, and to study their physicochemical properties including binding abilities toward guest molecules. In this report, the synthesis of cyclic hexa-, octa- and decapeptides containing alanine, aspartic acid or lysine together with glycine is described. The temperature dependence of the conformation of these cyclic peptides in solution was studied by using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy with a view to elucidating fundamental features of cyclic peptides required for host molecule activity.

Results and Discussion

Synthesis of Cyclic Peptides

Cyclic hexa-(I—IV, VI), octa-(VII—X) and decapeptides (XI) were synthesized by the cyclization of the corresponding linear precursors. Cyclodimerization was applied for the preparation of a cyclic hexa-peptide (V). The structures of the cyclic peptides are given in Chart 1. Cyclization of linear peptides was carried out through the azide(–N_3), *p*-nitrophenyl ester(–ONp), and/or *N*-hydroxysuccinimide ester(–ONSu) derivatives. Diethyl phosphorocyanidate (DEPC)[4)] was also used as a cyclization reagent. In order to avoid racemization during the cyclization reaction, linear precursors were so designed that a glycine

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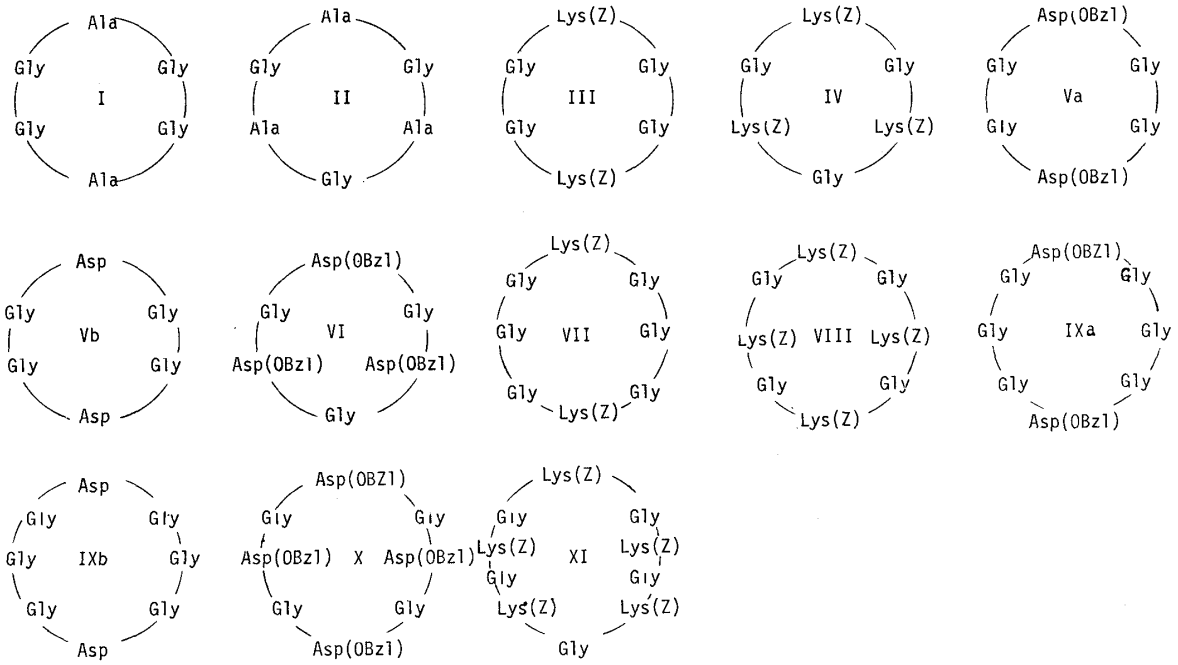

Chart 1

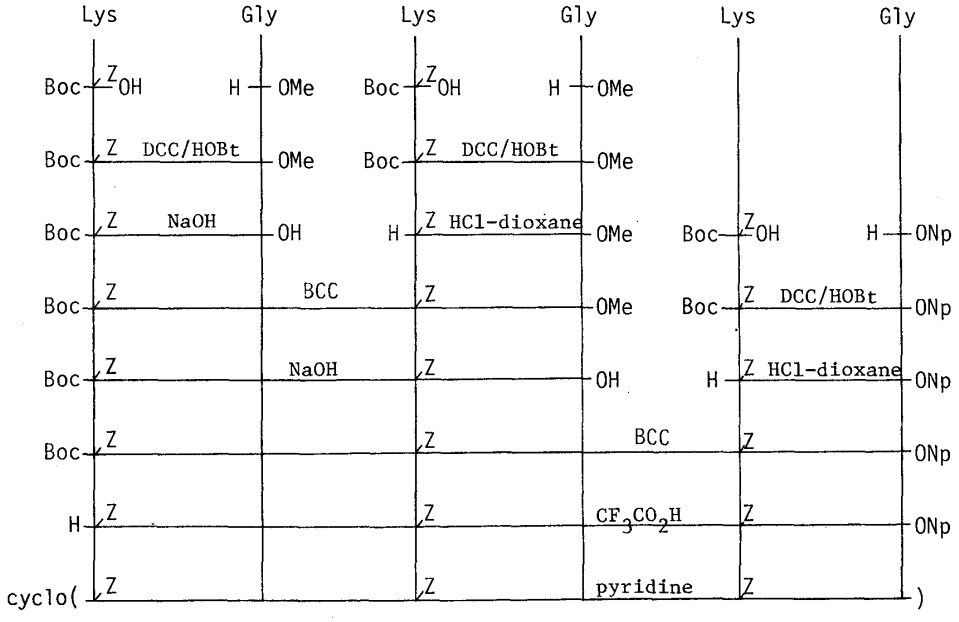

Chart 2

residue is situated at the C-terminal end; the synthetic route to cyclo(–Lys(Z)–Gly–)$_3$ (IV) is shown in Chart 2 as a typical example. The reaction yields in cyclization under various conditions were studied in the case of III, and these results are summarized in Table I. The cyclization yields for the other peptides are also listed though they were not optimized. The cyclization methods used did not greatly affect the reaction yield. The cyclization yield decreased with increasing concentration of starting materials, probably due to the formation of polymeric substances by intermolecular reaction, but the extent of the yield decrease was not significant at 10^{-3} M, a concentration suitable for practical purposes. In the cyclization reactions of the linear precursors, no cyclic dodeca-, hexadeca- or eicosapeptides formed by

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cyclodimerization reaction were detected. However, in the synthesis of Va starting from tripeptide, intermolecular cyclodimerization predominated. The reaction yield was almost the same as those of the intramolecular cyclization reactions starting from linear hexapeptides. This is considered to be due to the stabilized conformation of the reaction product, since it was proposed by Kopple *et al.* that the conformation of cyclic hexapeptides such as Va is fixed by 1,4-transannular hydrogen bonds.[5] Preparation of the cyclic peptide III from tripeptide *p*-nitrophenyl ester, tripeptide azide or hexapeptide *p*-nitrophenyl ester as the starting material was reported by Izumiya *et al.*[6] The reactions were carried out under conditions analogous to

TABLE I. Reaction Yields at the Cyclization Stage

Method[a]	Concentration (mM)	Isolation yield (%)										
		I	II	III	IV	VII	VIII	Va	VI	IXa	X	XI
N_3	0.43	60[c]		68								
	1.1	57[c]	50[c]	64								
	10	35[c]	40[c]	50								
ONp[b]	0.43	40	62	59	40	48	40					
	1.1	42	63	55	35	41	35					36
	10	25	37	35	21	22	21					
ONSu[b]	0.43			60	35	41						
	1.1			57	29	35	29					
	10			37	20		20					
DEPC	0.43			62	30	27						
	1.1			56	25	25	26					
	10			38	14	18	14					
Others								51[d]	33[e]	40[e]	60[e]	

a) Cyclization methods: N_3, acid azide in pyridine medium;, ONp, *p*-nitrophenyl ester in pyridine medium; ONSu, *N*-hydroxysuccinimide ester in pyridine; DEPC, diethyl phosphorocyanidate in dimethylformamide. *b*) Reactions were carried out using a microfeeder and the concentrations listed are the final ones after complete addition. *c*) Water was used instead of pyridine. *d*) Cyclodimerization of tripeptide *p*-nitrophenyl ester in pyridine using a microfeeder. Final concentration of the *p*-nitrophenyl ester added was 2.5 mM. *e*) ONp method (final concentration of 2.8 mM).

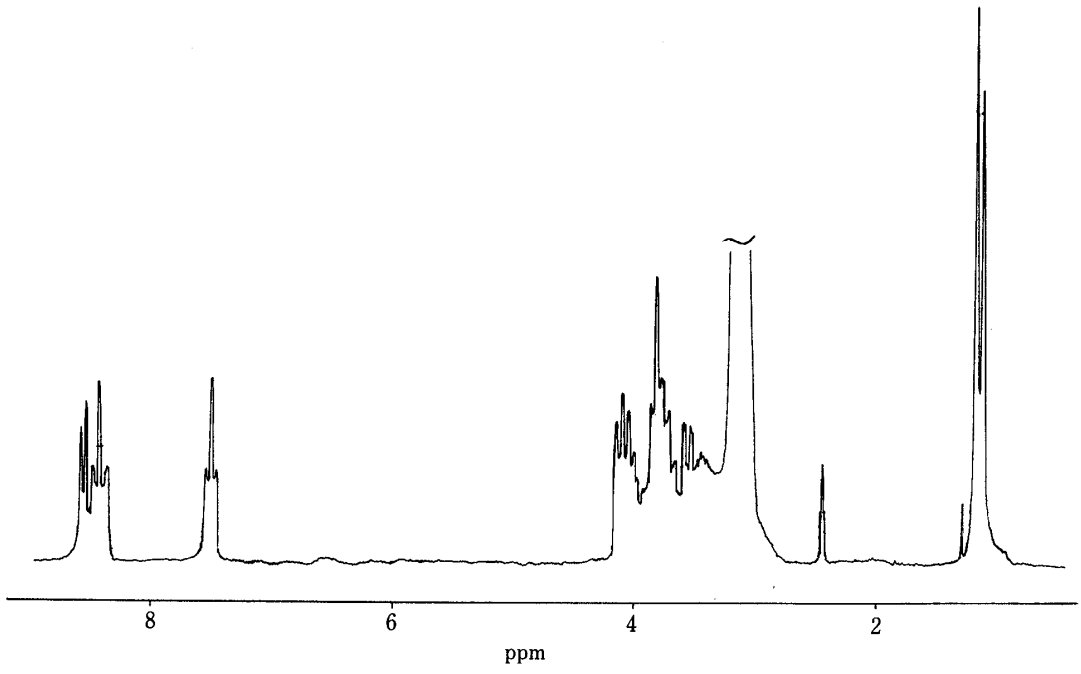

Fig. 1. The 200 MHz ^{1}H-NMR Spectrum of I in DMSO-d_6 at 30 °C

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TABLE II. Physical

Compound	I	II	III	IV	Va	Vb
mp (dec.)	300 °C	300 °C	258—260 °C[b]	240—242 °C	256—258 °C[b]	240 °C[b]
Recryst. solv.[a]	A	D–E	A–W	N–W	D–W–E	A–W
FD-MS *m/e*	370 (M^+)	384 (M^+)	775 ($M^+ + 23$)	958 ($M^+ + 23$)	638 (M^+)	459 ($M^+ + 1$)
$[\alpha]_D^{20}$	−6.3°	−5.5°	−13.5°[b]	−16.0°	−31.8°[b]	−5.8°
(conc., solv.)[a]	(*c*=0.3, D)	(*c*=0.1, D)	(*c*=2, D)	(*c*=2, D)	(*c*=1, D)	(*c*=0.5, W)
IR: ν_{max}^{Nujol} cm^{-1}	3300 (ν_{NH}) 1650 ($\nu_{C=O}$) 1540 (δ_{NH})	3300 (ν_{NH}) 1670 ($\nu_{C=O}$) 1540 (δ_{NH})	3300 (ν_{NH}) 1690 ($\nu_{C=O}$) 1650 ($\nu_{C=O}$) 1560 (δ_{NH})	3300 (ν_{NH}) 1680 ($\nu_{C=O}$) 1650 ($\nu_{C=O}$) 1560 (δ_{NH})	3300 (ν_{NH}) 1660 ($\nu_{C=O}$) 1640 ($\nu_{C=O}$) 1550 (δ_{NH})	3300 (ν_{NH}) 1730 ($\nu_{C=O}$) 1650 ($\nu_{C=O}$) 1540 (δ_{NH})
Formula	$C_{14}H_{22}N_6O_6$	$C_{15}H_{24}N_6O_6$	$C_{36}H_{48}N_8O_{10}$	$C_{48}H_{63}N_9O_{12}$		
Analysis (%)						
Calcd	C, 45.40 H, 5.99 N, 22.69	C, 46.87 H, 6.29 N, 21.87	C, 57.44 H, 6.43 N, 14.88	C, 60.18 H, 6.63 N, 13.16		
Found	C, 45.15 H, 6.00 N, 22.48	C, 46.83 H, 6.31 N, 21.79	C, 57.41 H, 6.56 N, 14.75	C, 60.09 H, 6.59 N, 13.16		
^{1}H-NMR: δ in DMSO-d_6 200 MHz	1.22 (6H, d), 3.40—3.80 (8H, m), 4.10—4.22 (2H, m), 7.53 (2H, t), 8.40 (2H, t), 8.47 (2H, d)	1.23 (9H, d), 3.69—3.72 (6H, m), 4.15—4.20 (3H, m), 7.98 (3H, d), 8.24 (3H, t)	1.10—1.80 (12H, m), 2.80—3.10 (4H, m), 3.60—3.80 (8H, m), 3.90—4.10 (2H, m), 4.99 (4H, s), 7.22 (2H, br), 7.33 (10H, s), 7.48 (2H, t), 8.39 (2H, d), 8.45 (2H, t)	1.10—1.80 (18H, m), 2.80—3.20 (6H, m), 3.60—3.85 (6H, m), 4.00—4.20 (3H, m), 4.99 (6H, s), 7.33 (15H, s), 7.20—7.26 (3H, br), 7.95 (3H, d), 8.29 (3H, t)	2.65—3.02 (4H, m), 3.50—3.90 (8H, m), 4.42—4.56 (2H, m), 5.12 (4H, s), 7.38 (10H, s), 7.63 (2H, t), 8.38 (2H, t), 8.75 (2H, d)	2.65—2.95 (4H, m), 3.55—3.92 (8H, m), 4.40—4.56 (2H, m), 7.63 (2H, t), 8.36 (2H, t), 8.73 (2H, d), 12.33 (2H, br)

a) A, ethanol; D, dimethylformamide; W, water; N, acetonitrile; E, ether; S, dimethylsulfoxide. *b*) Reported values for III,[6]

ours, and the reported reaction yield (16—41%) is compatible with ours. The physical characteristics of the cyclic peptides (I—XI) are listed in Table II.

Conformational Analysis of the Peptide Backbone by ^{1}H-NMR Spectroscopy

^{1}H-NMR spectroscopy is considered to be one of the most useful tools for conformational analysis of peptides, since it provides information on the conformational state of peptides in solution.[8,9] In this study, the temperature dependency of the NH proton signals of cyclic peptides was examined in the range of 30—120 °C. The ^{1}H-NMR spectrum of cyclo(–Gly–Ala–Gly–)$_2$ (I) is shown in Fig. 1. Signals at 7.5—8.5 ppm were assigned as NH protons. It is clear that I is composed of three kinds of magnetically nonequivalent amino acid residues and one of them gives a signal at fairly high field (δ 7.5 ppm). It can be concluded that I exists in a conformation with C_2-symmetry, that the two Gly–Ala–Gly fragments are physicochemically identical and that one residue per fragment is in an unusual environment.

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Characteristics of Cyclic Peptides

VI	VII	VIII	IXa	IXb	X	XI
223—224 °C A–W 787 ($M^+ + 1$)	265—267 °C A–W 867 ($M^+ + 1$)	220—224 °C N–W 1278 ($M^+ + 1$)	250 °C A–W 752 (M^+)	224—226 °C E–A–W 573 ($M^+ + 1$)	256—258 °C S–W 1049 (M^+)	250—255 °C N–W 1596 (M^+)
−13.5° (c=1, D)	−12.5° (c=1, D)	−10.0° (c=1, D)	−17.0° (c=0.5, A–W)	−16.1° (c=1, W)	−27.6° (c=1, D)	−9.0° (c=1, D)
3250 (ν_{NH}) 1720 ($\nu_{C=O}$) 1665 ($\nu_{C=O}$) 1550 (δ_{NH})	3300 (ν_{NH}) 1700 ($\nu_{C=O}$) 1660 ($\nu_{C=O}$) 1540 (δ_{NH})	3300 (ν_{NH}) 1680 ($\nu_{C=O}$) 1650 ($\nu_{C=O}$) 1540 (δ_{NH})	3300 (ν_{NH}) 1730 ($\nu_{C=O}$) 1650 ($\nu_{C=O}$) 1550 (δ_{NH})	3300 (ν_{NH}) 1700—1640 ($\nu_{C=O}$) 1550 (δ_{NH})	3250 (ν_{NH}) 1720 ($\nu_{C=O}$) 1700 ($\nu_{C=O}$) 1630 ($\nu_{C=O}$) 1550 (δ_{NH})	3300 (ν_{NH}) 1690 ($\nu_{C=O}$) 1660 ($\nu_{C=O}$) 1550 (δ_{NH})
$C_{39}H_{42}N_6O_{12}\cdot H_2O$	$C_{40}H_{54}N_{10}O_{12}$	$C_{64}H_{84}N_{12}O_{16}$	$C_{34}H_{40}N_8O_{12}$	$C_{20}H_{28}N_8O_{12}\cdot H_2O$	$C_{52}H_{56}N_8O_{16}\cdot H_2O$	$C_{90}H_{105}N_{15}O_{20}\cdot H_2O$
C, 58.13 H, 5.50 N, 10.43 C, 58.32 H, 5.29 N, 10.55	C, 55.42 H, 6.28 N, 16.16 C, 55.40 H, 6.30 N, 16.20	C, 60.18 H, 6.63 N, 13.16 C, 59.95 H, 6.60 N, 13.46	C, 54.25 H, 5.36 N, 14.89 C, 54.19 H, 5.34 N, 14.76	C, 40.67 H, 5.12 N, 18.98 C, 40.38 H, 4.94 N, 18.72	C, 58.53 H, 5.48 N, 10.50 C, 58.54 H, 5.34 N, 10.62	C, 58.85 H, 6.60 N, 12.87 C, 58.57 H, 6.64 N, 13.13
2.58—3.00 (6H, m), 3.54—4.20 (6H, m), 4.40—4.50 (3H, m), 5.07 (6H, s), 7.33 (15H, s), 8.11 (3H, t), 8.19 (3H, d)	1.10—1.80 (12H, m), 2.80—3.10 (4H, m), 3.50—4.00 (12H, m), 4.00—4.10 (2H, m), 4.99 (4H, s), 7.33 (10H, s), 7.21 (2H, br), 7.97—8.21 (8H, m)	1.10—1.80 (24H, m), 2.80—3.10 (8H, m), 3.40—4.00 (8H, m), 4.00—4.20 (4H, m), 4.99 (8H, s), 7.33 (20H, s), 7.20—7.24 (4H, br), 7.80—8.00 (4H, m), 8.05—8.25 (4H, m)	2.61—3.01 (4H, m), 3.52—3.95 (12H, m), 4.45—4.55 (2H, m), 5.07 (4H, s), 7.30 (10H, s), 7.79 (2H, t), 8.01 (2H, t), 8.43 (2H, t), 8.45 (2H, d)	2.58—2.80 (4H, m), 3.62—3.82 (12H, s), 4.35—4.62 (2H, m), 7.78 (2H, t), 8.01 (2H, t), 8.43 (2H, t), 8.45 (2H, d)	2.50—3.02 (8H, m), 3.48—4.00 (8H, m), 4.44—4.64 (4H, m), 5.10 (8H, s), 7.33 (20H, s), 8.13 (4H, t), 8.21 (4H, d)	1.10—1.80 (30H, m), 2.80—3.00 (10H, m), 3.30—4.00 (10H, m), 4.10—4.30 (5H, m), 4.99 (10H, s), 7.33 (25H, s), 7.18—7.25 (5H, br), 7.85—8.30 (10H, m)

$[\alpha]_D$ −13.5° (c=2, D), mp 263—265 °C; for Va,[7] $[\alpha]_D$ −33.1° (c=1, D), mp 256 °C; for Vb,[7] mp 270 °C.

The situation is the same for III. The three amino acid residues magnetically discriminated are, therefore, Ala(or Lys(Z)), Gly acylated by Ala(or Lys(Z)) and Gly acylating Ala(or Lys(Z)). Signals of Gly NH and Ala(or Lys(Z)) NH are easily distinguished by their multiplicities, since Gly NH coupled with α-CH_2 to afford triplet signal while NH of Ala(or Lys(Z)) coupled with one α-CH to give a doublet. Signals of peptide amide protons were analyzed in the temperature range of 30—120 °C. Figure 2 shows that temperature dependence of NH signals of III. At 30 °C, the signal of one Gly NH appears at a higher field region than 8 ppm, and another Gly NH and Lys(Z) NH appear at lower field. The spectrum at 120 °C is different from that at 30 °C, as shown. The signals at lower field shifted to the higher field region, though those at higher field remained at the original positions. Similar results were also obtained for the cyclic peptides of general form cyclo(–Gly–Xxx–Gly–)$_2$, such as cyclo(–Gly–Ala–Gly–)$_2$ (I), cyclo(–Gly–Asp(OBzl)–Gly–)$_2$ (Va) and cyclo(–Gly–Asp–Gly–)$_2$ (Vb). A linear relationship was obtained within the temperature range of 30—

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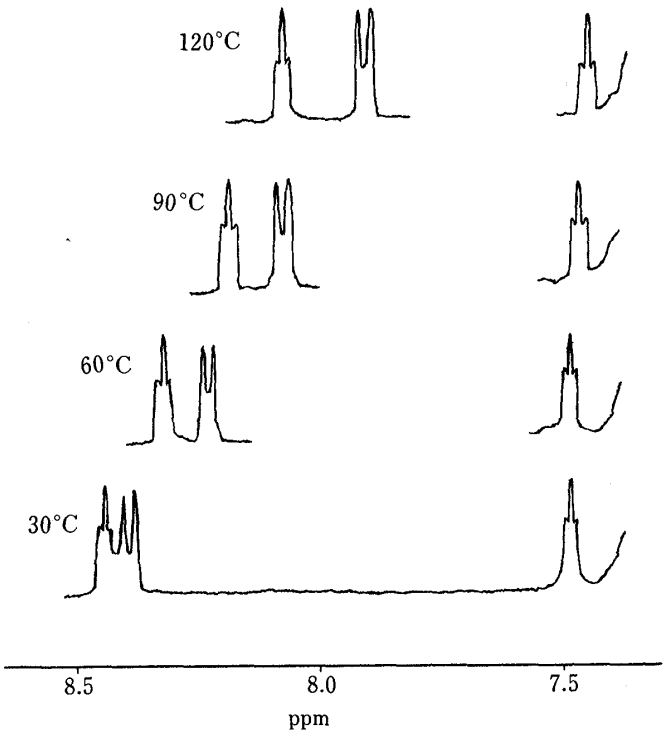

Fig. 2. Temperature Dependence of NH Signals of III in DMSO-d_6

TABLE III. Temperature Coefficients of Chemical Shift for Peptide Amide Protons

Cyclic peptide	Amino acid residue	$\Delta\delta/\Delta T^{a)}$ ($\times 10^3$)	Cyclic peptide	Amino acid residue	$\Delta\delta/\Delta T^{a)}$ ($\times 10^3$)
I	Gly	0.55	VII	Gly	2.66
	Ala	5.22		Gly	3.22
	Gly	4.77		Gly	3.55
II	Gly	3.78		Lys(Z)	2.66
	Ala	2.89	VIII	Gly	3.44
III	Gly	0.56		Lys(Z)	3.67
	Lys(Z)	4.89	IXa	Gly	1.45
	Gly	4.33		Gly	2.06
IV	Gly	4.44		Gly	4.80
	Lys(Z)	3.22		Asp(OBzl)	4.03
Va	Gly	0.33	IXb	Gly	1.65
	Gly	4.83		Gly	2.27
	Asp(OBzl)	5.90		Gly	4.40
Vb	Gly	0.00		Asp	4.00
	Gly	4.83	X	Gly	3.67
	Asp	5.90		Asp(OBzl)	4.33
VI	Gly	3.03			
	Asp(OBzl)	3.33			

a) ppm shift to higher field per degree.

120 °C when the values of NH signals in δ (ppm) were plotted as a function of temperature. Values of temperature coefficient $\Delta\delta/\Delta T$ were determined from the slope (Table III).

In the measurement of the linear hexapeptide, the trifluoroacetate of H–(Gly–Lys(Z)–Gly)$_2$–OH (**31**), it was found that all the NH signals appeared at around 8.4 ppm at 30 °C and were temperature-dependent ($\Delta\delta/\Delta T = 4.8$—$5.6 \times 10^{-3}$). This observation suggests that two Gly residues of cyclic peptides of the type cyclo(–Gly–Xxx–Gly–)$_2$ are involved in in-

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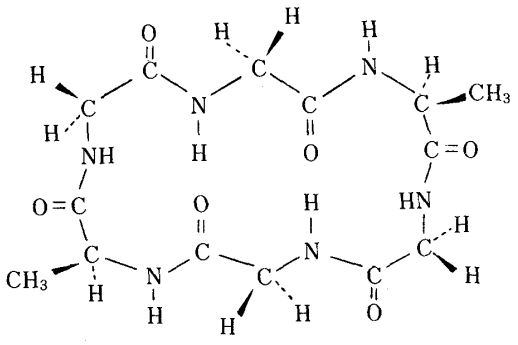

Fig. 3. Estimated Structure of the Cyclic Hexapeptide I in DMSO Solution

tramolecular hydrogen bonding, since the chemical shift of the NH proton (triplet) is temperature-independent. This effect was not observed in the linear peptides. Measurements of compounds I and III in TFA resulted in the appearance of all NH signals in the narrow range of 7.8—8.0 ppm, and all were temperature-dependent. This suggests that the intramolecular hydrogen bonds observed in DMSO solution do not exist in TFA (which has strong solvating power) because of the preponderance of intermolecular hydrogen bonding with the solvent. Our results are in accord with those reported by Kopple *et al.*,[5,10] who proposed that cyclic hexapeptides such as cyclo(–Gly–Tyr–Gly–)$_2$ and cyclo(–Gly–Leu–Gly–)$_2$, two glycyl peptide amide protons are hydrogen-bonded (1,4-transannular hydrogen bonds) and the other peptide amide protons are exposed to the solvent. Following the conformational proposals of Kopple *et al.*,[5] based on a ^{1}H-NMR study of deuterium-labeled peptides, it is assumed that the two glycine residues which are involved in the hydrogen bonding are those which acylate alanine in I. Similarly, the glycine residues which acylate lysine or aspartic acid may be involved in the hydrogen bonding in the case of III or V, respectively. Summarizing the above results, the structure of I in DMSO solution is assumed to be as shown in Fig. 3.

In contrast, looser conformations were assumed for compounds other than I, III and V. The spectrum of II shows that each of three glycine residues and each of three alanine residues are in identical environments. At 120 °C both signals shifted to higher field with $\Delta\delta/\Delta T \times 10^3$ values of 3.8 and 2.9 for glycine and alanine residues, respectively (Table III). Similar results were obtained for IV and VI. The values were determined to be 4.4 (Gly) and 3.2 (Lys(Z)) for IV and 3.0 (Gly) and 3.3 (Asp(OBzl)) for VI, as shown in Table III. These values are intermediate between those observed for a residue exposed to the solvent and those for a hydrogen-bonded residue. It is assumed that cyclic hexapeptides in the general form cyclo(–Gly–Xxx–)$_3$ are not fixed in a rigid conformation by means of hydrogen bonding but rather are in a flexible state where the C_3-symmetry of the peptide sequence is reflected in the three-dimensional structure. Within the region of δ 7.78—8.45, three triplet signals due to Gly NH and one doublet due to Asp(or Lys) NH were observed in the spectra of the cyclic octapeptides VII, IXa and IXb. As shown in Table III, the temperature dependencies of these signals were different from each other. The values of $\Delta\delta/\Delta T \times 10^3$ for these cyclic peptides are considerably smaller than those of corresponding linear octapeptides ($\Delta\delta/\Delta T \times 10^3 = 4.8$—5.5). It may be concluded, therefore, that VII and IX are loosely fixed in part through weak intramolecular hydrogen bonds and the interaction is not as tight as that of the cyclic hexapeptides I, III and V, for which a rigid structure with C_2-symmetry is assumed. In the spectra of cyclic octapeptides VIII and X, only two kinds of NH signals are observed at lower field (δ 7.80—8.25), and they are both strongly temperature-dependent. The structures of these compounds may be rather flexible, as in the case of IV and VI. Unfortunately, the cyclic decapeptide XI could not be analyzed because of its low solubility.

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TABLE IV. Preparation and Physical Constants of Linear Peptides

Compound	Synthetic procedure[a]	Starting material[b]	Yield %	mp °C (Rec. solv.[c])	$[\alpha]_D^\circ$ (c, solv.[c])	Analysis[d]
Z–Gly–Ala–Gly–OMe (**1**)	a	**74**,[11] **75**[12]	81	120—120.5 (M–E)	−35.5 (c=1, D)	Element
Z–(Gly–Ala–Gly)$_2$–OMe (**2**)	a	**1** (after c), **76**[13]	78	218—220 (M)	−11.7 (c=1, D)	Element
Z–(Gly–Ala–Gly)$_2$–NHNH$_2$ (**3**)	b	**2**	85	228—231 (M)	−34.0 (c=1, H)	IR
2HCl·H–(Gly–Ala–Gly)$_2$–NHNH$_2$ (**4**)	c	**3**	96	Amorphous solid		IR
Boc–Ala–Gly–ONp (**5**)	d	**77**,[14] **78**[15]	70	132—134 (C–E)	−20.6 (c=1, D)	Element
HCl·H–Ala–Gly–ONp (**6**)[21]	e	**5**	96	Hygroscopic powder		IR
Z(OMe)–Gly–Ala–Gly–ONp (**7**)	a	**79**,[14] **6**	65	173—174 (N–E)	−12.6 (c=1, D)	Element
HCl·H–Gly–Ala–Gly–ONp (**8**)	e	**7**	97	Hygroscopic powder		IR
Z–(Gly–Ala–Gly)$_2$–ONp (**9**)	a	**76**,[13] **8**	70	235—237 (N–W)	−12.6 (c=1, D)	Element
HBr·H–(Gly–Ala–Gly)$_2$–ONp (**10**)	f	**9**	80	Hygroscopic powder		IR
Z–(Ala–Gly)$_2$–OMe (**11**)	a	**80**,[16] **75**[12]	80	186—187 (M–E)	−10.5 (c=1, M)	Element
HCl·H–(Ala–Gly)$_2$–OMe (**12**)	c	**11**	95	Hygroscopic powder		IR
Z–(Ala–Gly)$_3$–OMe (**13**)	a	**80**,[16] **12**	65	228 (dec.) (M)		IR
Z–(Ala–Gly)$_3$–NHNH$_2$ (**14**)	b	**13**	90	270 (dec.) (M)	—	IR
2HCl·H–(Ala–Gly)$_3$–NHNH$_2$ (**15**)	c	**14**	85	Hygroscopic powder		IR
Boc–(Ala–Gly)$_2$–ONp (**16**)	a	**81**,[16] **6**	65	215—217 (N–E)	−4.9 (c=1, D)	Element
TFA·H–(Ala–Gly)$_2$–ONp (**17**)	g	**16**	95	Hygroscopic powder		IR
Boc–(Ala–Gly)$_3$–ONp (**18**)	a	**81**,[16] **17**	60	235 (H–N)	−2.5 (c=0.5, D)	Element
TFA·H–(Ala–Gly)$_3$–ONp (**19**)	g	**18**	Quant	Hygroscopic powder		IR
HCl·H–Gly–Lys(Z)–Gly–OBzl (**20**)	e	**82**[6]	90	Hygroscopic powder		IR
Z(OMe)–(Gly–Lys(Z)–Gly)$_2$–NHNH$_2$ (**21**)	b	**95**[6]	90	198—201 (D–E)	−6.6 (c=1, D)	Element
2TFA·H–(Gly–Lys(Z)–Gly)$_2$–NHNH$_2$ (**22**)	h	**21**	90	Hygroscopic powder		IR
Boc–Lys(Z)–Gly–ONp (**23**)	d	**83**,[17] **78**[15]	60	98—99 (P–O)	−10.4 (c=1, D)	Element
HCl·H–Lys(Z)–Gly–ONp (**24**)	e	**23**	90	Hygroscopic powder		IR
Z(OMe)–Gly–Lys(Z)–Gly–ONp (**25**)[6]	a	**79**,[14] **24**	70	151—153 (N)	−12.2 (c=1, D)	Element
HCl·H–Gly–Lys(Z)–Gly–ONp (**26**)	e	**25**	90	Hygroscopic powder		IR
Z(OMe)–(Gly–Lys(Z)–Gly)$_2$–ONp (**27**)[6]	a	**84**,[6] **26**	80	171—173 (N)	−12.2 (c=1, D)	Element
TFA·H–(Gly–Lys(Z)–Gly)$_2$–ONp (**28**)[6]	h	**27**	95	Hygroscopic powder		IR
Z(OMe)–(Gly–Lys(Z)–Gly)$_2$–ONSu (**29**)	i	**85**[6]	85	152—154 (W–N)	—	IR
TFA·H–(Gly–Lys(Z)–Gly)$_2$–ONSu (**30**)	h	**29**	Quant	Hygroscopic powder		IR
TFA·H–(Gly–Lys(Z)–Gly)$_2$–OH (**31**)	h	**85**[6]	97	Hygroscopic powder		IR
Boc–Lys(Z)–Gly–OMe (**32**)[22]	d	**86**,[17] **90**[18]	70	Oil	—	IR
Boc–Lys(Z)–Gly–OH (**33**)	j	**32**	92	Oil	—	IR
HCl·H–Lys(Z)–Gly–OMe (**34**)	e	**32**	90	Hygroscopic powder		IR
Boc–(Lys(Z)–Gly)$_2$–OMe (**35**)	a	**33**, **34**	88	107—109 (O–E)	−10.2 (c=1, D)	Element
Boc–(Lys(Z)–Gly)$_2$–OH (**36**)	j	**35**	90	73—75 (O–E)	−5.4 (c=1, D)	Element
Boc–(Lys(Z)–Gly)$_3$–ONp (**37**)	a	**36**, **24**	66	168—170 (N)	−5.5 (c=1, D)	Element
TFA·H–(Lys(Z)–Gly)$_3$–ONp (**38**)	h	**37**	98	Hygroscopic powder		IR

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Boc–(Lys(Z)–Gly)$_3$–OMe (**39**)	a	**34**, **36**	86	181—183 (N)	−9.8 (c=1, D)	Element
Boc–(Lys(Z)–Gly)$_3$–OH (**40**)	j	**39**	95	156—158 (W–N)	−6.8 (c=1, D)	Element
Boc–(Lys(Z)–Gly)$_3$–ONSu (**41**)	i	**40**	70	141—143 (N)	—	IR
TFA·H–(Lys(Z)–Gly)$_3$–ONSu (**42**)	h	**41**	90	Hygroscopic powder		IR
TFA·H–(Lys(Z)–Gly)$_3$–OH (**43**)	h	**40**	90	Hygroscopic powder		IR
Boc–Asp(OBzl)–Gly–ONp (**44**)	a	**87**,[13] **78**[15]	86	136—137 (P–O)	—	Element
Boc–Gly–Asp(OBzl)–Gly–ONp (**45**)	a	**44** (after e), **88**[14]	88	80—82 (O–P)	−19.3 (c=1, D)	IR
Boc–Asp(OBzl)–Gly–OH (**46**)	k	**89**,[19] H–Gly–OH	Quant	Oil	—	IR
Boc–(Asp(OBzl)–Gly)$_2$–ONp (**47**)	a	**44** (after e), **46**	69	150 (O–P)	—	Element
Boc–(Asp(OBzl)–Gly)$_3$–ONp (**48**)	a	**47** (after e), **46**	84	115—125 (O–P)	—	Element
Z(OMe)–Gly–Lys(Z)–Gly$_2$–OMe (**49**)	a	**84**,[6] **90**[18]	78	167—169 (N)	−6.5 (c=1, D)	Element
Z(OMe)–Gly–Lys(Z)–Gly$_2$–ONp (**50**)	a	**84**,[6] **78**[15]	82	153—155 (N)	−1.3 (c=1, D)	IR
Z(OMe)–Gly–Lys(Z)–Gly$_2$–OH (**51**)	j	**49**	72	115—118 (N)	—	IR
HCl·H–Gly–Lys(Z)–Gly$_2$–ONp (**52**)	e	**50**	85	Amorphous solid		IR
Z(OMe)–(Gly–Lys(Z)–Gly$_2$)$_2$–ONp (**53**)	a	**51**, **52**	78	210 (dec.) (N–W)	−5.4 (c=0.5, D)	Element
TFA·H–(Gly–Lys(Z)–Gly$_2$)$_2$–ONp (**54**)	h	**53**	90	Hygroscopic powder		IR
HCl·H–Gly–Lys(Z)–Gly$_2$–OMe (**55**)	e	**49**	65	Hygroscopic powder		IR
Z(OMe)–(Gly–Lys(Z)–Gly$_2$)$_2$–OMe (**56**)	a	**51**, **55**	79	214—217 (N)	−7.5 (c=0.5, D)	Element
Z(OMe)–(Gly–Lys(Z)–Gly$_2$)$_2$–OH (**57**)	j	**56**	85	260 (dec.) (N–W)	—	Element
Z(OMe)–(Gly–Lys(Z)–Gly$_2$)$_2$–ONSu (**58**)	i	**57**	85	200—205 (N)	—	IR
TFA·H–(Gly–Lys(Z)–Gly$_2$)$_2$–ONSu (**59**)	h	**58**	95	Hygroscopic powder		IR
TFA·H–(Gly–Lys(Z)–Gly$_2$)$_2$–OH (**60**)	h	**57**	95	Hygroscopic powder		IR
Boc–(Lys(Z)–Gly)$_4$–ONp (**61**)	a	**40**, **91**[12]	80	197—200 (N–W)	−6.0 (c=1, D)	Element
TFA·H–(Lys(Z)–Gly)$_4$–ONp (**62**)	h	**61**	90	Hygroscopic powder		IR
Boc–(Lys(Z)–Gly)$_4$–OMe (**63**)	a	**40**, **92**[17]	90	190—192 (W–M)	−10.5 (c=0.5, D)	Element
Boc–(Lys(Z)–Gly)$_4$–OH (**64**)	j	**63**	75	220—222 (W–N)	−16.9 (c=1, D)	Element
Boc–(Lys(Z)–Gly)$_4$–ONSu (**65**)	i	**64**	80	240 (dec.) (W–N)	—	Element
TFA·H–(Lys(Z)–Gly)$_4$–ONSu (**66**)	h	**65**	90	Hygroscopic powder		IR
TFA·H–(Lys(Z)–Gly)$_4$–OH (**67**)	h	**64**	90	Amorphous solid		IR
Boc–Gly$_2$–Asp(OBzl)–Gly–OH (**68**)	a	**93**[7] (after e), **94**[14]	91	151—152 (O–P)	−15.4 (c=1, D)	Element
Boc–Gly$_2$–Asp(OBzl)–Gly–ONp (**69**)	a	**45** (after e), **94**[14]	76	131—132 (O)	−19.5 (c=1, D)	Element
Boc–(Gly$_2$–Asp(OBzl)–Gly)$_2$–ONp (**70**)	a	**69** (after e), **68**	88	240 (dec.) (O–P)	−25.0 (c=1, D)	Element
Boc–(Asp(OBzl)–Gly)$_4$–ONp (**71**)	a	**48** (after e), **46**	35	170—172 (O–P)	—	Element
Boc–(Lys(Z)–Gly)$_5$–ONp (**72**)	a	**64**, **24**	65	224 (dec.) (N–W)	—	Element
TFA·H–(Lys(Z)–Gly)$_5$–ONp (**73**)	h	**72**	Quant	Amorphous solid	—	IR

a) Procedures a—k are described in the text. *b*) **74**: Z–Gly–OH; **75**: HCl·H–Ala–Gly–OMe; **76**: Z–Gly–Ala–Gly–OH; **77**: Boc–Ala–OH; **78**: HBr·H–Gly–ONp; **79**: Z(OMe)–Gly–OH; **80**: Z–Ala–Gly–OH; **81**: Boc–Ala–Gly–OH; **82**: Z(OMe)–Gly–Lys(Z)–Gly–OBzl; **83**: Boc–Lys(Z)–OH; **84**: Z(OMe)–Gly–Lys(Z)–Gly–OH; **85**: Z(OMe)–(Gly–Lys(Z)–Gly)$_2$–OH; **86**: Boc–Lys(Z)–OH; **87**: Boc–Asp(OBzl)–OH; **88**: Boc–Gly–OH; **89**: Boc–Asp(OBzl)–ONSu; **90**: HCl·H–Gly–OMe; **91**: HCl·H–Lys(Z)–Gly–ONp; **92**: HCl·H–Lys(Z)–Gly–OMe; **93**: Boc–Gly–Asp(OBzl)–Gly–OH; **94**: Boc–Gly–OH; **95**: Z(OMe)–(Gly–Lys(Z)–Gly)$_2$–OBzl. *c*) Abbreviations of solvents are: M (methanol), E (ether), D (dimethylformamide), W (water), H (0.2 N HCl), C (acetone), N (acetonitrile), P (petroleum ether) and O (ethyl acetate). *d*) Element: elemental analysis (for C, H and N) showed less than 0.3% deviation from calculated values. IR: infrared spectrum consistent with the structure.

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Experimental

All melting points were determined on a Yamato MP-21 melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a JASCO IR-1, optical rotations on a JASCO DIP-4 digital polarimeter, and field desorption mass spectra (FD-MS) on a JEOL JMS-01SG-2. ^{1}H-NMR spectra were recorded on a JEOL JNM-FX 100 (100 MHz) or JNM-FX 200 (200 MHz) with tetramethylsilane (TMS) as an internal standard. Analysis of temperature dependency was carried out at the peptide concentration of 10^{-2} M in DMSO-d_6 or TFA with the JNM-FX 200 instrument equipped with a VTS-2 temperature control unit.

Synthesis of Linear Peptides

Linear precursor peptides for cyclization and their intermediate peptides were synthesized by the usual liquid-phase method as summarized in Table IV. Typical synthetic procedures (a—k) are described below.

Procedure a: Z–Gly–Ala–Gly–OMe (1)——BCC (7.4 ml, 56 mmol) was added to a solution of Z–Gly–OH[11] (11.7 g, 56 mmol) and Et_3N (7.8 ml, 56 mmol) in THF under stirring at −20 °C, and stirring was continued for 10 min. Next, a solution of HCl·H–Ala–Gly–OMe[12] (11.0 g, 56 mmol) and Et_3N (7.8 ml, 56 mmol) in THF (200 ml) was added, and the mixture was stirred for 4 h at −10 °C then for 10 h at room temperature. After evaporation of the solvent, the residue was dissolved in chloroform (300 ml), washed successively with water, 10% HCl, water, 4% sodium bicarbonate and saturated NaCl, then dried over Na_2SO_4. The solution was evaporated to dryness and the residue was recrystallized.

Procedure b: Z–Gly–Ala–Gly$_2$–Ala–Gly–NHNH$_2$ (3)——Hydrazine hydrate (3.6 ml, 76 mmol) was added to a solution of **2** (4 g, 7.6 mmol) in MeOH (50 ml) and the mixture was refluxed for 1 h, then allowed to stand for 1 h at room temperature. The precipitate was filtered off. This product was combined with a second crop obtained from the concentrated mother liquor, and recrystallized.

Procedure c: 2HCl·H–Gly–Ala–Gly$_2$–Ala–Gly–NHNH$_2$ (4)——A solution of **3** (526 mg, 1 mmol) in MeOH (50 ml) and 2 N HCl (2 ml) was subjected to catalytic hydrogenation over 10% Pd/carbon (50 mg). After evaporation of the solvent, the residue was crystallized.

Procedure d: Boc–Ala–Gly–ONp (5)——DCC (4.1 g, 20 mmol) was added to a solution of Boc–Ala–OH[14] (3.7 g, 20 mmol), HBr·H–Gly–ONp[15] (5.8 g, 20 mmol), HOBt (2.7 g, 20 mmol) and Et_3N (2.8 ml, 20 mmol) in THF (200 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and at room temperature for 15 h. Dicyclohexylurea was filtered off and the filtrate was evaporated *in vacuo*. The residue was dissolved in ethyl acetate and the solution was washed with 10% citric acid; water, 4% sodium bicarbonate, water and finally saturated sodium chloride solution. The solution was evaporated and the residue was recrystallized.

Procedure e: HCl·H–Ala–Gly–ONp (6)——A solution of **5** (3.67 g, 10 mmol) in dioxane (5 ml) was treated with 4 N HCl–dioxane (5 ml), and the mixture was stirred at room temperature. After addition of ether, the resulting powder was collected and dried over NaOH pellets.

Procedure f: HBr·H–Gly–Ala–Gly$_2$–Ala–Gly–ONp (10)——**9** (643 mg, 1 mmol) was treated with 25% HBr in AcOH (5 ml) at room temperature and ether was added. The resulting powder was collected and dried over NaOH pellets.

Procedure g: TFA·Ala–Gly–Ala–Gly–ONp (17)——**16** (4.9 g, 10 mmol) was treated with TFA (20 ml) at room temperature. The solution was then evaporated *in vacuo*, and the residue was triturated with ether.

Procedure h: 2TFA·H–Gly–Lys(Z)–Gly$_2$–Lys(Z)–Gly–NHNH$_2$ (22)——**21** (50 mg, 0.05 mmol) was treated with TFA–anisole (2 ml–0.01 ml) in an ice-bath at 0 °C as described above.

Procedure i: Z(OMe)–Gly–Lys(Z)–Gly$_2$–Lys(Z)–Gly–ONSu (29)——*N*-Hydroxysuccinimide (43 mg, 0.37 mmol) and EDC·HCl (57 mg, 0.3 mmol) were added to a solution of Z(OMe)–Gly–Lys(Z)–Gly$_2$–Lys(Z)–Gly–OH[6] (233 mg, 0.25 mmol) in DMF (5 ml) at 0 °C. The reaction mixture was stirred at room temperature for 48 h. The solvent was evaporated off *in vacuo*, water was added to the residue; the resulting powder was collected by filtration, and recrystallized.

Procedure j: Boc–Lys(Z)–Gly–OH (33)——**32** (4.51 g, 10 mmol) was dissolved in MeOH (50 ml) and 1 N NaOH (12 ml) at 0 °C. The solution was allowed to stand for 2 h at room temperature. After evaporation of the solvent *in vacuo* and addition of water, the solution was acidified with 10% citric acid under cooling to yield a yellow oil.

Procedure k: Boc–Asp(OBzl)–Gly–OH (46)——A solution of Boc–Asp(OBzl)–ONSu[19] (12.6 g, 30 mmol) in THF (30 ml) was added to a solution of H–Gly–OH (2.25 g, 30 mmol) and $NaHCO_3$ (5.04 g, 60 mmol) in water (30 ml) and the mixture was stirred at room temperature for 1 h. The THF was evaporated off *in vacuo*, and the resulting aqueous solution was acidified with 1 N HCl then extracted with ethyl acetate. The combined extracts were washed with water and saturated NaCl, and dried over Na_2SO_4. The solvent was evaporated off to give an oil, which was used directly in the following procedure.

General Procedures for the Preparation of Cyclic Peptides

Azide Method——The dihydrochloride or ditrifluoroacetate of peptide hydrazide (**4**, **15**, or **22**) was treated with an equimolar amount of sodium nitrite in the presence of hydrochloric acid at 0 °C. The progress of the reaction was monitored by means of the hydrazine test.[20] Cyclization was initiated by pouring the azide solution into an

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appropriate amount of sodium bicarbonate solution (0.01 M) and the mixture was kept for 2 d at 0 °C. For the synthesis of cyclo(–Gly–Lys(Z)–Gly–)$_2$ (III), pyridine was used instead of aqueous bicarbonate solution. After reducing the reaction mixture to 2—3 ml *in vacuo*, the concentrate was subjected to ion-exchange chromatography (Dowex 1 and Dowex 50). The neutral fraction passing through both columns was recrystallized from an appropriate solvent.

Activated Ester Method——A solution of a salt of a peptide *p*-nitrophenyl ester or *N*-hydroxysuccinimide ester (**10**, **19**, **28**, **30**, **38**, **42**, **54**, **59**, **62**, **66**, or **73**) in DMF (0.01 M) was added dropwise to pyridine using a microfeeder over a period of 4 h. The amount of pyridine was such that it gave the final concentration shown in Table I. The reaction was carried out at room temperature in the case of *N*-hydroxysuccinimide esters or at 60 °C for *p*-nitrophenyl esters. The isolation procedure for the cyclic peptide was the same as that in the case of the azide method.

DEPC Method——A dimethylformamide solution of the TFA salt of a peptide acid (**31**, **43**, **60** or **67**) at an appropriate concentration was neutralized with an equimolar amount of triethylamine, and 1.2 eq of DEPC was added. The resulting cyclic peptide was isolated in the same manner as above.

Cyclodimerization——Boc–Gly–Asp(OBzl)–Gly–ONp **45** (558 mg, 1 mmol) was treated with 4 N HCl in dioxane (3 ml), and the mixture was kept for 30 min at room temperature. The precipitated HCl·H–Gly–Asp(OBzl)–Gly–ONp was collected. The precipitate was dissolved in DMF (15 ml) containing acetic acid (0.1 ml) and added dropwise to pyridine (400 ml) using a microfeeder at 60 °C over a period of 4 h. The reaction mixture was kept at room temperature overnight. After removal of the solvent, ethanol was added. The resulting solid was crystallized from DMF–water–ether to give Va; yield 235 mg (51%), mp 256—258 °C, $[\alpha]_D^{20} = -31.8°$ ($c=1$, DMF), FD-MS *m/e* 638 (M^+). The procedure is basically identical to that reported by Sugihara *et al.*[7] except for use of the microfeeder. The isolation yield of Va was reported to be 40% under their conditions.

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References and Notes

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2) The amino acid residues mentioned in this paper are of the L-configuration. The abbreviations used to denote amino acid derivatives and peptides are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **11**, 1726 (1972). Other abbreviations used: Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Bzl = benzyl, Np = *p*-nitrophenyl, N_3 = azide, NSu = *N*-hydroxysuccinimidyl, Boc = *tert*-butyloxycarbonyl, DEPC = diethyl phosphorocyanidate, DCC = *N*,*N*′-dicyclohexylcarbodiimide, BCC = isobutyl chloroformate, TFA = trifluoroacetic acid, THF = tetrahydrofuran, DMF = dimethylformamide, DMSO = dimethylsulfoxide, HOBt = *N*-hydroxybenzotriazole, EDC = *N*-ethyl-*N*′-dimethylaminopropyl-carbodiimide.
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