Conformational Studies of Bis(cyclic tetrapeptide), *S,S'*-Bis[cyclo-(Gly-L-hemiCys-Sar-L-Pro)]. A Proposed Castanet-type Structure in a Dimethyl Sulfoxide Solution

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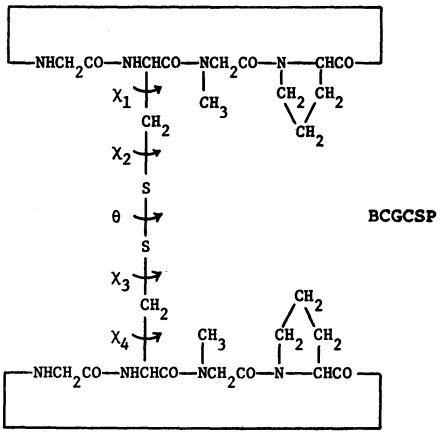
(Received February 1, 1982)

A bis(cyclic tetrapeptide), *S,S'*-bis[cyclo(Gly–L-hemiCys–Sar–L-Pro)] (BCGCSP), was prepared by coupling two homodetic cyclotetrapeptide. The conformations of BCGCSP in solutions were investigated by means of NMR, Raman, and CD spectroscopy. The ^{1}H-NMR data in dimethyl-d_6 sulfoxide (Me_2SO-d_6) demonstrated that the two cyclic skeletons of BCGCSP take the same conformations as that of an authentic cyclic tetrapeptide, cyclo-[Gly–L-Cys(Bzl(OMe))–Sar–L-Pro] (CGCSP). The ^{1}H-NMR spectrum of CGCSP contains three sets of resonances, corresponding to three different molecular conformations. From the Raman lines of BCGCSP at 529 cm^{-1} and 683 cm^{-1} in Me_2SO-d_6, it is deduced that the two HC_α–$C_\beta S$ dihedral angles are 180°(trans), while one of the SS–$C_\beta C_\alpha$ dihedral angles is 20—30° and the other is either near 90° or near 180°. In addition, the values of two Raman lines predict that the $C_\beta S$–SC_β dihedral angle is in the range of near 90°. Such a conformation around the $C_\beta S$–SC_β bond is also reasonable in the light of the presence of a CD transition (CS–SC) at $\lambda > 250$ nm in water. By combining the above data, the CPK molecular model of BCGCSP was constructed. An inspection of the model reveals that this bis(cyclic tetrapeptide) is likely to adopt a "castanet-type" structure in Me_2SO-d_6. At the same time the individual cyclic skeleton is considered to take three different ring conformations in equilibrium.

Synthetic cyclic peptides are useful model compounds for elucidating the conformational features (for example, β-turns, γ-turns, and transannular hydrogen bonds) which are recognized as important in protein structure.[1,2] They are also ideal candidates for a new class of carrier molecules which have the capability of ion-binding and ion-transporting through a biological membrane,[3-5] for it is artificially possible to synthesize cyclic peptides, which are molecularly designed to be furnished with the structural characteristics of naturally occurring peptidic substances.[6] Some of the cyclic tetrapeptides have been investigated as to their solution conformations, in connection with their residual properties.[7] However, our cation-binding study of cyclotetrapeptide, cyclo(L-Pro–Sar)$_2$ demonstrated that an efficient ion-dipole interaction was unlikely to take place for lack of conformational flexibility.[8] This result also implied that cyclic peptide comprised of a relatively small number of amino acid residues (4—5) tends to form 2 : 1 (peptide=2, cation=1) complexes with some metal cations.

Thus, a bis(cyclic tetrapeptide), *S,S'*-bis[cyclo(Gly–L-hemiCys–Sar–L-Pro)], in which two homodetic cyclotetrapeptides are joined by a covalent bond, was designed and synthesized. The methodology for such a synthesis of a bis(cyclic peptide) was established by Schwyzer *et al.*[9] and Murakami *et al.*[10] Schwyzer *et al.* synthesized a *S,S'*-bis[cyclo(Gly–L-hemiCys–Gly–Gly–L-Pro)] and proposed a possible model of a cation pump by this peptide, which is driven by the oxidoreduction process. Murakami *et al.* synthesized a *S,S'*-bis-[cyclo(Gly–L-hemiCys–Gly–L-His–L-Cap–L-Und)] and found that this peptide enhanced the hydrolysis of *p*-nitrophenyl carboxylates bearing a long alkyl chain. The conformational study of the bis(cyclic tetrapeptide) described herein provided spectroscopic evidence that this peptide takes a "castanet-type" structure in Me_2SO-d_6. This structure is requisite for all available carbonyl oxygens to bind to a cation cooperatively. During the present study, our attention was focused on the restriction of the total molecular conformation by the $C_\alpha C_\beta SSC_\beta C_\alpha$ group.



BCGCSP

As has been mentioned above, BCGCSP is a useful model compound containing an SS linkage for elucidating conformational features in naturally occurring peptides. However, the very poor solubility of this peptide restricted the use of a variety of solvents. Therefore, dimethyl sulfoxide was chosen as the solvent for the NMR and Raman studies, and water was utilized for the CD study.

Results and Discussion

Conformations of Individual Cyclic Peptide Skeletons of the Bis(cyclic tetrapeptide) in Solution. The 360 MHz ^{1}H-NMR spectrum of *S,S'*-bis[cyclo(Gly–L-hemiCys–Sar–L-Pro)] (BCGCSP) in Me_2SO-d_6 is shown in Fig. 1. More or less resolved signals for each proton in BCGCSP are seen, particularly in the region of 0—6 ppm. At the same time, at least six amide NH proton signals are positioned at 6.8—8.8 ppm. At first, the characterization of the amide NH protons was inferred from variable-temperature ^{1}H-NMR studies. The temperature effect

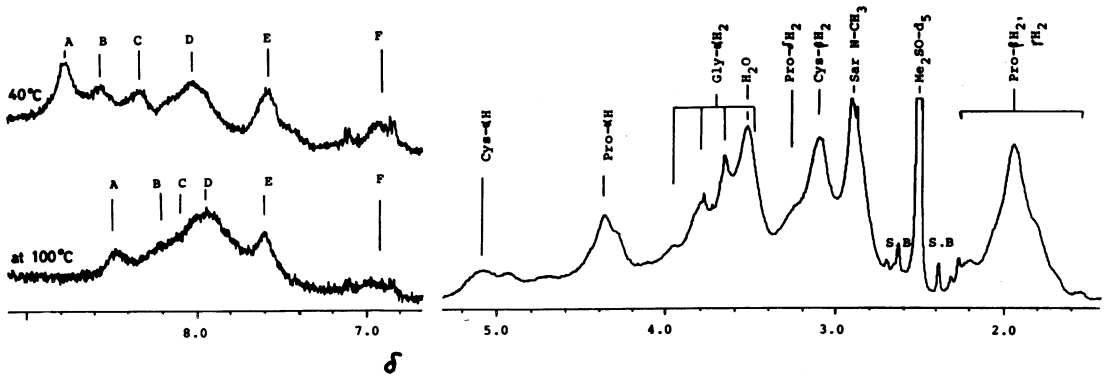


Fig. 1. ^{1}H-NMR spectrum (360 MHz) of BCGCSP in Me_2SO-d_6 at 100 °C and (upper left) at 40 °C. Concentration: 10 g dm^{-3}, S.B.: spinning sideband.

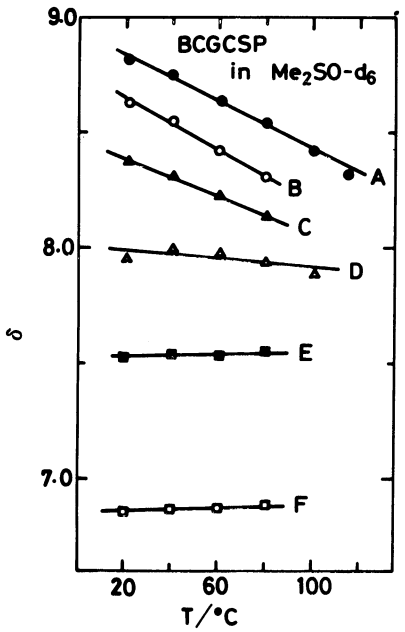


Fig. 2. Temperature dependence of the Gly and Cys amide proton chemical shifts of BCGCSP in Me_2SO-d_6

on the chemical shift of NH signals in Me_2SO-d_6 is shown in Fig. 2. Three NH resonance signals (A, B, and C) move upfield as the temperature increases [$|d\delta(dT)^{-1}|>0.004$ ppm deg^{-1}], while the other three resonances (D, E, and F) are almost independent of the temperature [$|d\delta(dT)^{-1}|<0.001$ ppm deg^{-1}]. These remarkable differences in temperature dependence are attributed to the accessibility of the amide NH proton to a solvent [an internal NH proton (buried or hydrogen-bonded) or an external one (exposed to the solvent)]. According to the usual criteria of accessibility determined from temperature dependence, the three NH protons (A, B, and C) must be exposed to the solvent, while the other three NH protons (D, E, and F) must be shielded from the solvent. A comparison of the ^{1}H-NMR spectrum of the authentic monocyclic tetrapeptide with that of the bis(cyclic tetrapeptide) will now give helpful information for the assignment of the NH proton signals of BCGCSP. The 360 MHz ^{1}H-NMR spectrum of the authentic cyclic tetrapeptide, cyclo[Gly–L-Cys(Bzl(OMe))–Sar–L-Pro] (CGCSP), in Me_2SO-d_6 is shown in Fig. 3. Assignments of signals for each proton were made by spin-decoupling. The NMR parameters of the NH proton resonances of BCGCSP and CGCSP

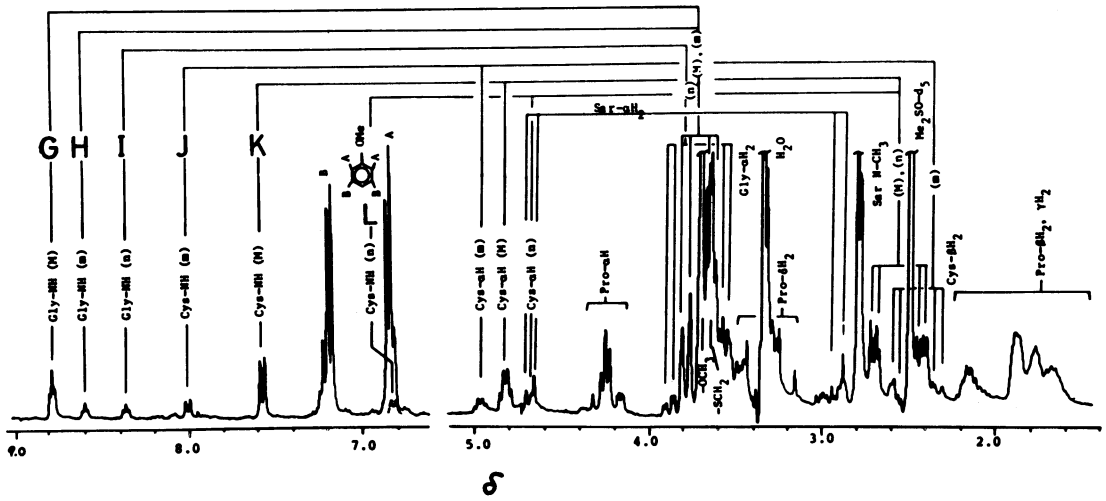


Fig. 3. ^{1}H-NMR spectrum (360 MHz) of CGCSP in Me_2SO-d_6 at 20 °C. Concentration: 25 g dm^{-3}.

TABLE 1. AMIDE PROTON RESONANCES AND CONFORMATIONAL PARAMETERS OF BCGCSP AND CGCSP IN Me_2SO-d_6 AT 20 °C

BCGCSP		CGCSP					
δ[a]	$d\delta(dT)^{-1}\times10^3$/ppm deg^{-1}		δ[a]	$d\delta(dT)^{-1}\times10^3$/ppm deg^{-1}	$J_{NH-C_\alpha H}$/Hz	θ/deg	ϕ/deg
8.81(A)	−5.00		8.79(G)	−4.13	5.8, 5.0	170, 70	130
8.63(B)	−5.83	Gly-NH	8.60(H)	−4.73	6.4, 5.1	167, 73	133
8.37(C)	−4.08		8.36(I)	−3.80	6.4, 6.0	167, 47	107
7.96(D)	−0.92		8.01(J)	−0.00	9.5	180	−120
7.53(E)	0.33	Cys-NH	7.58(K)	−0.07	9.7	180	−120
6.86(F)	0.50		≈6.82(L)	≈0	10.1	180	−120

a) From internal tetramethylsilane (TMS). b) The nomenclature used for the conformational dihedral angle, ϕ, is that set forth by the IUPAC-IUB Commission on Biochemical Nomenclature, 1970; see *Biochemistry*, **9**, 3471 (1970). c) $\theta=\phi\pm60°$. d) Calculated from observed $\sum J_{NH-C_\alpha H}$ using a Ramachandran relationship, $\sum J_{NH-C_\alpha H}=7.9[\cos^2(\phi+60°)+\cos^2(\phi-60°)]-1.55[\cos(\phi+60°)+\cos(\phi-60°)]-1.35[\sin^2(\phi+60°)+\sin^2(\phi-60°)]=6.0\cos^2\phi-1.5\cos\phi+12.5\sin^2\phi$. e) Calculated from observed $J_{NH-C_\alpha H}$ using a Ramachandran relationship, $J_{NH-C_\alpha H}=7.9\cos^2\theta-1.55\cos\theta-1.35\sin^2\theta$. f) $\phi=\theta+60°$.

TABLE 2. PARAMETERS FOR THE THREE DIFFERENT CONFORMERS (**M**, **m**, AND **n**) OBSERVED IN A SOLUTION OF CGCSP IN Me_2SO-d_6 AT 21°C[a]

Residue	Type of NMR spectrum	Conformation		
		M	**m**	**n**
Gly	ABX	$\delta_A=3.65, J_{AB}=-15.7$ $\delta_B=3.80, J_{AX}=5.8$ $\delta_X=8.79, J_{BX}=5.0$	$\delta_A=3.65, J_{AB}=-11.2$ $\delta_B=3.80, J_{AX}=6.4$ $\delta_X=8.60, J_{BX}=5.1$	$\delta_A=3.56, J_{AB}=-11.4$ $\delta_B=3.89, J_{AX}=6.4$ $\delta_X=8.36, J_{BX}=6.0$
Cys(Bzl(OMe))[b]	ABMX	$\delta_A=2.44, J_{AB}=-13.5$ $\delta_B=2.72, J_{AM}=8.2$ $\delta_M=4.84, J_{BM}=5.7$ $\delta_X=7.58, J_{MX}=9.7$	$\delta_A=2.37, J_{AB}=-13.5$ $\delta_B=2.60, J_{AM}=8.2$ $\delta_M=4.98, J_{BM}=5.7$ $\delta_X=8.01, J_{MX}=9.5$	$\delta_A=2.37, J_{AB}=-17.0$ $\delta_B=2.60, J_{AM}=5.1$ $\delta_M=4.69, J_{BM}=5.9$ $\delta_X=6.82, J_{MX}=10.1$
Sar	A_3PQ	$\delta_A=2.81, J_{PQ}=-16.6$ $\delta_P=2.92$ $\delta_Q=4.69$	$\delta_A=2.79$	$\delta_A=2.78$
Pro	ABCDPQX	$\delta_A=$, $\delta_B=$, $\delta_C=$ 1.55—1.95 $\delta_D=2.05$—2.25 $\delta_P=$, $\delta_Q=$ 3.32 $\delta_X=4.26$	$\delta_A=$, $\delta_B=$, $\delta_C=$ 1.55—1.95 $\delta_D=2.05$—2.25 $\delta_P=$, $\delta_Q=$ 3.25—3.50 $\delta_X=4.16$—4.34	$\delta_A=$, $\delta_B=$, $\delta_C=$ 1.55—1.95 $\delta_D=2.05$—2.25 $\delta_P=$, $\delta_Q=$ 3.25—3.50 $\delta_X=4.16$—4.34

a) Chemical shift, δ, coupling constant, J/Hz. The coupling constants, J, are to within ±0.5 Hz. b) Signals for the protons of the *p*-methoxybenzyl group were omitted.

in Me_2SO-d_6 are summarized in Table 1. Each of the chemical shifts and each of the temperature coefficients of the three NH resonances (A, B, and C) of BCGCSP indicate good similarities to those of the three NH resonances (G, H, and I) of CGCSP. As to the parameters of the remaining three proton resonances (D, E, and F), they also closely resemble those of three resonances (J, K, and L) of CGCSP. This finding supports the idea that three resonances (A, B, and C) are to be assigned to the glycyl NH protons, and the other resonances (D, E, and F), to the cystyl NH ones. It also leads us to the conclusion that the two homodetic cyclic peptide skeletons of BCGCSP take the same structure as that of monocyclic peptide, despite the fact that two cyclic peptides are joined by a bridge containing the S–S bond. Therefore, it seems plausible that the two cyclic skeletons exist in the same conformational state. These conclusions imply that three pairs of the glycyl and cystyl NH proton (A and E, B and D, and C and F) are to be ascribed to the three coexisting structures of BCGCSP in equilibrium. It is of great interest that the conformational properties of the cyclic skeleton of this bis(peptide) are essentially determined by the ring conformation of the individual cyclic tetrapeptide.

Our discussion will, therefore, be concentrated on multiple-ring conformations of CGCSP in Me_2SO-d_6. The ^{1}H-NMR spectrum of CGCSP contains three sets of resonances for each proton. The chemical shifts for the three sets of signals and corresponding coupling constants are summarized in Table 2. All the coupling constants were experimentally obtained. The computer-simulated spectrum using these spectral parameters was in good agreement with the observed one. The N-CH_3 protons of the sarcosyl residue in CGCSP give rise to three signals, at 2.81, 2.79, and 2.78 ppm, with the relative intensity of 3 : 1 : 1 (*cf.* Fig. 4(8)). This, together with the occurrence of the three NH proton signals of each glycyl and cysteinyl residue, means that

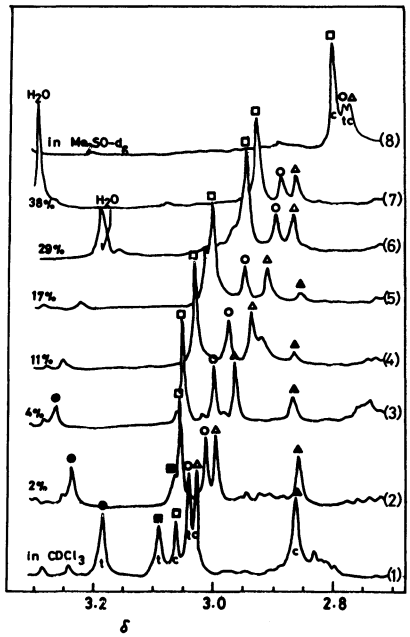


Fig. 4. ^{1}H-NMR spectrum (360 MHz) of N–CH_3 proton resonances of CGCSP in $CDCl_3$–Me_2SO-d_6 mixture at 20 °C.
Me_2SO-d_6 volume fraction: (1) 0, (2) 0.02, (3) 0.04, (4) 0.11, (5) 0.17, (6) 0.29, (7) 0.38, (8) 1.0. The signal having same origin is indicated by the same mark.

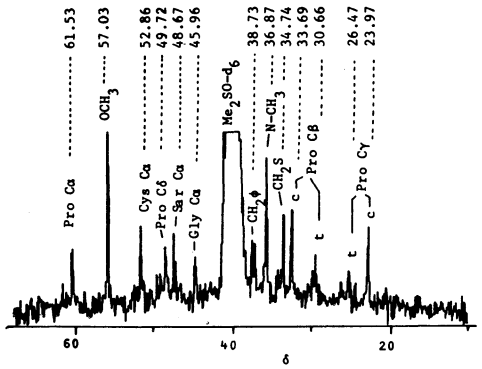
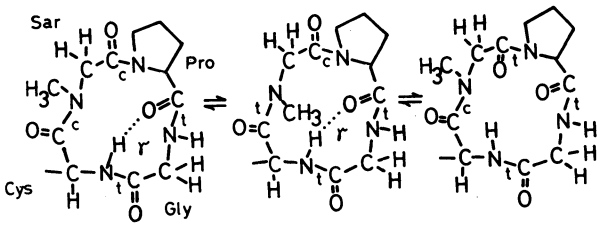


Fig. 5. ^{13}C-NMR spectrum (90 MHz) of CGCSP in Me_2SO-d_6 at 21 °C.
Concentration: 30 g dm^{-3}



M **m** **n**

Fig. 6. Three different conformations (**M**, **m**, and **n**) of CGCSP proposed on ground of the NMR data in Me_2SO-d_6.

CGCSP has three conformational states. When benzene-d_6 (C_6D_6) was added to a chloroform-d_1 ($CDCl_3$) solution of CGCSP, all the N–CH_3 proton signals represented two different tendencies of upfield shifts. Figure 4(1) shows the 360 MHz ^{1}H-NMR spectrum of CGCSP in $CDCl_3$. The addition of C_6D_6 produced large upfield shifts for the signal at 3.18(●), 3.09(■), and 3.04(○), and small upfield shifts for the remaining signals at 3.06(□), 3.02(△), and 2.86 ppm (▲). Taking into account this phenomenon, N–CH_3 protons anti to the carbonyl oxygen of its peptide bond (*i.e.*, the trans Cys–Sar peptide unit) can be differentiated from that in the cis peptide bond. That is, this differentiation technique is based upon the large benzene-induced upfield shift of the resonance ascribable to the N–CH_3 protons involved in the trans peptide bond.[11] It is thus concluded that the signals at 3.18(●), 3.09(■), and 3.04 ppm (○) are attributable to the N–CH_3 protons involved in the trans Cys–Sar peptide bond, while the other signals, at 3.06(□), 3.02(△), and 2.86 ppm (▲), are attributable to the N–CH_3 protons on the cis Cys–Sar bond in $CDCl_3$. The interaction of CGCSP with C_6D_6 in $CDCl_3$ will be discussed in detail in the following paper.[12] Figure 4(2)—4(8) indicates the ^{1}H-NMR spectral change in CGCSP in the sarcosyl N–CH_3 region when Me_2SO-d_6 was added to the $CDCl_3$ solution. It can be seen that three signals in $CDCl_3$, at 3.18(●), 3.09(■), and 2.86 ppm (▲), disappear gradually as the proportion of Me_2SO-d_6 increases. Finally, this solvent-titration study showed that the two N–CH_3 signals at 2.81 and 2.78 ppm in Me_2SO-d_6 are ascribable to the N–CH_3 protons on the cis Cys–Sar peptide bond (Fig. 4(8)). In addition, one remaining signal, at 2.79 ppm, can be attributed to the N–CH_3 protons on the trans Cys–Sar bond in Me_2SO-d_6. From the relative area underneath the N–CH_3 proton signals, it is also clear that a predominant conformer, **M**, has a cis Cys–Sar peptide bond, while two remaining minor conformers, **m** and **n**, contain a trans and a cis Cys–Sar peptide bonds respectively.

The chemical shifts of the Pro C_β and Pro C_γ carbon are often applicable in establishing the cis-trans isomeric state of the X–Pro peptide bond (X=any amino acid residue). The 90 MHz ^{13}C-NMR spectrum of CGCSP in Me_2SO-d_6 is shown in Fig. 5. From the literature information[13] about the differentiation of the trans X–Pro peptide bond from the cis X–Pro bond, it can be determined that the Pro C_β resonance at 33.7 ppm and the Pro C_γ resonance at 24.0 ppm are typical of the cis Sar–Pro peptide bond. Another Pro C_β resonance at 30.7 ppm and the Pro C_β resonance at 26.5 ppm can also be attributed to the trans Sar–Pro bond. As judged from the relative intensities of the Pro C_β and C_γ resonances, one-third of the populations of Sar–Pro peptide bonds seem to have the trans form. If one considers the cis-trans isomerism about the Cys–Sar and the Sar–Pro peptide bond, three of the four different conformations can be attributed to CGCSP, thus explaining all the situations mentioned above. At first, the glycyl and cysteinyl ϕ angles are calculated from the observed vicinal coupling constants, $J_{NH-C_\alpha H}$, using a Ramanchandran-type relationship (Table 1).[14] Taking these conformational parameters into account, three different CPK model of CGCSP can then be

constructed. Schematic representations of the proposed backbones for CGCSP in Me_2SO-d_6 are shown in Fig. 6. These three types of conformations indicate four characteristic features. First, the conformers, **M**, **m**, and **n**, contain cis-cis, trans-cis, and cis-trans peptide bond sequences of the Cys–Sar–Pro units respectively. Second, the conformation about the prolyl C_α–CO single bond is trans'(Pro $\psi=120°$)[15] in all three species. Third, a transannular hydrogen bond between the Cys amide NH proton and the Pro C=O oxygen would be possible. This satisfies the hydrogen-bonding requirement evidenced by the solvent shielding of the Cys NH proton. The molecular model indicates that the hydrogen bond is nonlinear, having a short C=O··· HN distance. In this respect, the rate of exchange of the amide NH protons in a mixed solvent of Me_2SO-d_6 and 10%-D_2O has been studied. The Gly amide NH proton in **M** was totally exchanged in less than 250 min at 30°C, while the NH proton of cystein in **M** remained unexchanged after 400 min. In **m**, the exchange of the glycyl NH proton was completed in less than 90 min, whereas the exchange of the cysteinyl NH proton took more than 150 min. These results provide strong evidence that the cysteinyl NH proton in at least two conformers (**M** and **m**) is involved in the formation of a transannular hydrogen bond. Fourth, similar species appear to arise in the conformation of CGCSP in acetonitrile-d_3 (CD_3CN), as will be discussed in the following paper.[12]

Conformations of a Side-chain Bridge Containing a Disulfide S–S Bond in Me_2SO-d_6. The Raman spectrum of BCGCSP in Me_2SO-d_6 is shown in Fig. 7. Two Raman lines are observed at 529 cm^{-1} due to a S–S stretching vibration and at 683 cm^{-1} due to a C–S stretching vibration, plus a line at 616 cm^{-1} derived from the solvent. The correlations of the S–S stretching frequencies, ν(S–S), with the conformational properties of the $C_\alpha C_\beta SSC_\beta C_\alpha$ fragment have so far been investigated on organic disulfides. Wart and Scheraga have studied the Raman spectra of a number of primary disulfides which are structurally related to cystine.[16] They proposed a correlation between ν(S–S) and the conformation about the C_β–S bonds in the $C_\alpha C_\beta SS_\beta C_\alpha$ fragment. According to their assumption, the observed value of ν(S–S) of 529 cm^{-1} can be ascribed to the conformation in which one SS–$C_\beta C_\alpha$ dihedral angle(χ_2) is 20—30° (neither cis nor gauche) and the other(χ_3) is 90° (neither gauche nor skew) or 180° (trans). In addition, the assignment of the C–S stretching modes was proposed by Sugeta *et al.*[17] It leads us to the conclusion that the value of ν(C–S) of 683 cm^{-1} is responsible for the conformation in which two HC_α–$C_\beta S$ dihedral angles (χ_1 and χ_4) are 180° (trans). As to the conformations about the S–S bond, compounds containing an unstrained $C_\alpha C_\beta SSC_\beta C_\alpha$ fragment generally have similar conformations (*i.e.*, $C_\beta S$–SC_β dihedral angles near ±90°). It will, then, be valid to say that the stereochemistry of the $C_\beta S$–SC_β group in BCGCSP has a restricted range of dihedral angles near ±90° in Me_2SO-d_6. This assumption is consistent with the results of the study of Wart and Scheraga.[16] They suggested that the observation of values of ν(S–S) higher than about 510 cm^{-1} would imply the presence of unstrained S–S bonds (*i.e.*, the dihedral angle retains its minimum energy value of ±90°).

Chirality of Disulfide S–S Bond in Water. Next, our discussion will be focused on the chirality of the disulfide bridge between two homodetic cyclic tetrapeptides. For the dimethyl sulfoxide solution, the circular dichroism (CD) spectrum was too complicated to analyze because of the presence of solvent absorption. Some information about the chirality was obtained, however, from the CD spectrum in water. The CD spectrum of BCGCSP is shown in Fig. 8: it has a small negative band ($[\theta]=-11$ deg cm^2 $dmol^{-1}$) at 295 nm, a positive band ($[\theta]=+1400$) at 253 nm, a large negative band ($[\theta]=-17700$) at 226 nm, and a positive band ($[\theta]=+4700$) at 208 nm. Taking into consideration the fact that BCGCSP does not contain any aromatic side chains, the 295 nm band and the 253 nm band can certainly be assigned to the disulfide chromophore. Moreover, the remaining two bands, at 226 nm and at 208 nm, are attributable to the amide-bond transition. The correlations between the chirality of the S–S bond and the

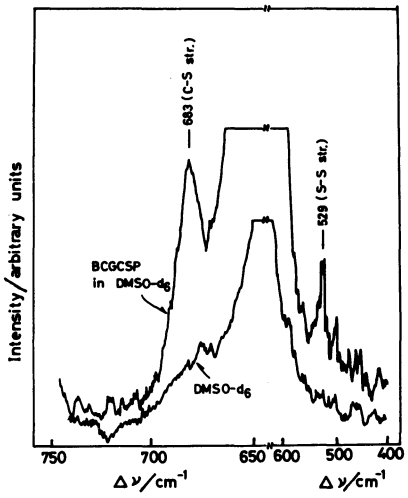


Fig. 7. Raman spectra of BCGCSP in Me_2SO-d_6 at room temperature.

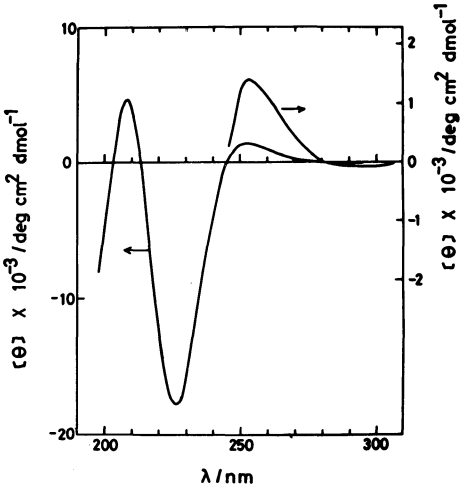


Fig. 8. CD spectrum of BCGCSP in H_2O at 25°C. Concentration: 2.26×10^{-4} mol dm^{-3}.

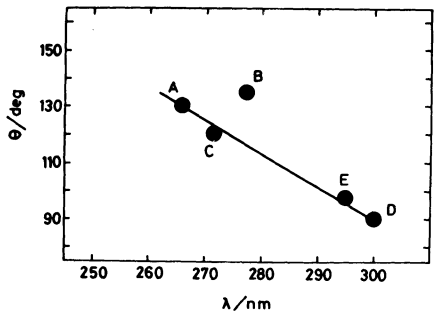


Fig. 9. Dependence of disulfide angle θ, to λ_{min} for various cyclic peptides containing S–S bond.
θ: The value which was estimated by CD spectra and/or measured on the molecular model. λ_{min}: Longer wavelength which affords a negative cotton effect and is attributed to the lowest energy transition. A: *S,S'*-Bis[cyclo(L-hemiCys–L-Pro)] in ethanol,[23] B: malformin-A in hexafluoro-1-propanol,[22] C: [2,7-L-Cystine]-gramicidin S in ethanol,[19] D: *S,S'*-bis[cyclo(Gly–L-hemiCys–Gly–Gly–L-Pro)] in 2,2,2-trifluoroethanol,[9] E: *S,S'*-bis[cyclo(Gly–L-hemiCys–Sar–L-Pro)] in H_2O.

sign of the two Cotton effects in the $250<\lambda<300$ nm and $230<\lambda<250$ nm regions were provided by the work of Carmack and Neubert.[18] According to it, a right-handed helical sence (*P*-chirality) is related to a positive Cotton effect at $\lambda>250$ nm and a negative one at $230<\lambda<250$ nm in the range of dihedral angles of $0°<\theta<90°$ [*vice versa* for a left-handed helical sense (*M*-chirality)]. The investigation of [2,7-L-Cystine]-gramicidin S provided an experimental finding concerning the chirality of the disulfide S–S bond.[19] It completed the evidence for the validity of the theoretical predictions formulated by Bergson[20] and by Linderberg and Michl.[21] If the 295 nm band of this bis(cyclic peptide) corresponds to the long-wavelength transition[20] of the disulfide S–S bond, the quadrant rule for a negative Cotton effect predicts a dihedral angle of either $90°<\theta<180°$ (*P*-chirality) or $-90°<\theta<0°$ (*M*-chirality). The S–S dihedral angles, θ, of compounds containing a cystine moiety previously reported are: (1) malformin-A[22] ($\theta=130$—$140°$, $[\theta]_{276-278}=-5500$ deg cm^2 dmol^{-1}), (2) [2,7-L-Cystine]-gramicidin S[19] ($\theta=120°$, $[\theta]_{271.5}=-7500$ in the difference spectrum between [2,7-L-Cystine]-gramicidin S and [2,7-S-acetamidomethyl-L-cysteine]-gramicidin S), and (3) *S,S'*-Bis[cyclo(Gly–L-hemiCys–Gly–Gly–L-Pro)][9] ($\theta=90°$). From these results, it can be seen that the lowest energy transition in the disulfide S–S chromophore occurs at the longer wavelength, as the S–S dihedral angle, θ, is reduced from 140° to 90° (Fig. 9). However, in the case of $\theta=90°$, the inherent optical activity could not be observed to determine whether the conformation around the S–S bond takes a *P*-chirality or not.[19] Actually, the CD spectrum of a bis(cyclic pentapeptide) shows no Cotton effect centered near 300 nm.[9] These findings substantiate a *P*-chirality with $\theta>90°$ for the S–S bond of BCGCSP. If we adopt the conclusion that the dihedral angle, θ, is 90°—100°, the observation of the negative Cotton effect at 295 nm agrees well with the rules of Linderberg and Michl[21] for the inherent optical

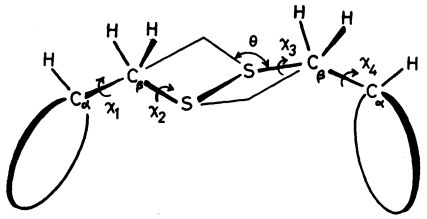


Fig. 10. Schematic representation of $C_\alpha C_\beta SSC_\beta C_\alpha$ moiety in BCGCSP.

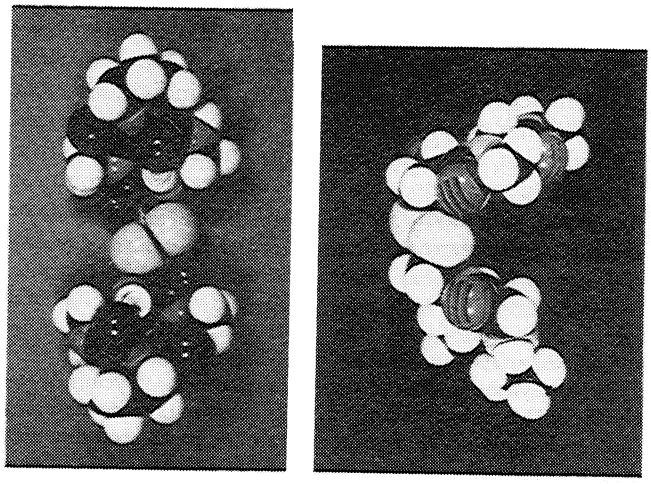


Fig. 11. Photograph of CPK model of proposed a "castanet-type" conformation of BCGCSP in solution. Individual ring conformation of BCGCSP represents that of species **M**, tentatively.

activity of the disulfide chromophore. A *P*-chirality with $\theta=90°$—$100°$ is also in better agreement with the linear correlation in Fig. 9 between the S–S dihedral angles and the corresponding transition wavelength. The 220—250 nm band could not be used to estimate the chirality of the disulfide bond, for it may contain contributions from perturbation by the peptide bond $n\rightarrow\pi^*$ transitions. On the other hand, the CD spectrum of monocyclic tetrapeptide, CGCSP, in water-methanol (9 : 1, by volume) ($[\theta]_{217}=-4700$ deg cm^2 dmol^{-1}) is remarkably different from that of BCGCSP. In the $n\rightarrow\pi^*$ transition region of amide bonds, the total contributions by the four amide-bond chromophores to the CD spectrum will be equal between CGCSP and BCGCSP. Therefore, the CD differences may originate from the perturbations between the amide chromophores of the two homodetic peptide rings in BCGCSP. In other words, the side-chain group of $C_\alpha C_\beta SSC_\beta C_\alpha$ in water is assumed to have such a conformation that they make one peptide ring approach to another one (Fig. 10).

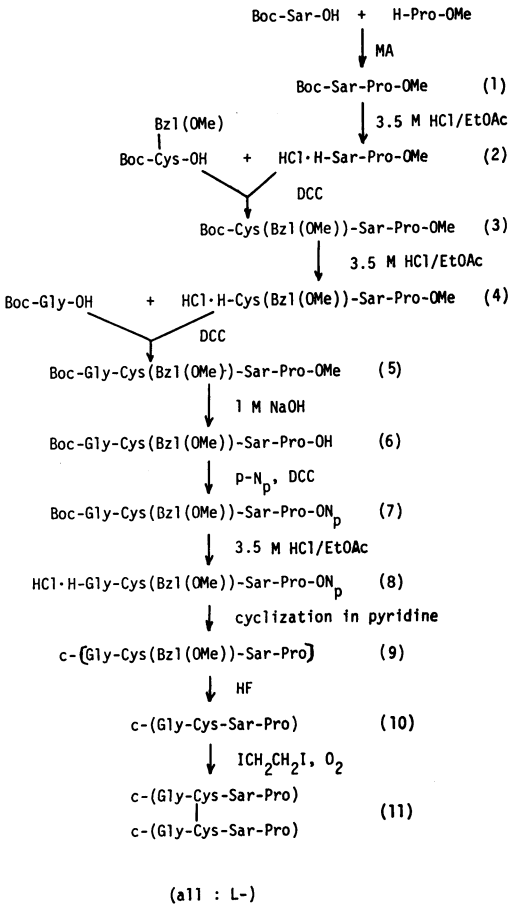
Molecular Conformation of BCGCSP in Me_2SO-d_6. The above assumption regarding the $C_\alpha C_\beta SSC_\beta C_\alpha$ structure in water seems to be reasonable in explaining the Raman spectrum in Me_2SO-d_6. The *P*-chirality of the S–S bond in BCGCSP will probably be retained even in Me_2SO-d_6, judging from the polarity of water and Me_2SO-d_6. Thus, a CPK model of the bis(cyclic tetra-

peptide) could be constructed so that the $C_\alpha\overset{\chi_1}{-}C_\beta\overset{\chi_2}{-}S\overset{\theta}{-}S\overset{\chi_3}{-}C_\beta\overset{\chi_4}{-}C_\alpha$ group was in the $\chi_1(180°)$-$\chi_2(20°—30°)$-$\theta(90°)$-$\chi_3(180°)$-$\chi_4(180°)$ conformation. At that time, the ring-part conformation of **M** was tentatively assumed to have the conformation of the constituent cyclic tetrapeptide. An inspection of the CPK model reveals that BCGCSP is likely to adopt a "castanet-type" structure, as is shown in Fig. 11. It should be noted that the two planes of the cyclic peptide rings are not essentially positioned in parallel fashion. Therefore, the proposed molecular conformation in the case is different from the specifically folded conformation proposed for *S,S'*-Bis-[cyclo(L-Cys–L-Pro)] in water.[24] The above considerations lead us to the conclusion that BCGCSP must have a "castanet-type" conformation in Me_2SO-d_6 as a whole of the molecule. At the same time, the individual cyclic peptide rings occur in the three interconverting species. This also led us to an interest in binding studies of this bis(cyclic peptide) to a metal ion. A detailed account on the cation-selectivity profiles of this peptide will be presented elsewhere.

Experimental

S,S'-Bis[cyclo(Gly–L-hemiCys–Sar–L-Pro)](BCGCSP)(11). This was synthesized according to Scheme 1. Cyclo[Gly–L-Cys(Bzl(OMe))–Sar–L-Pro](CGCSP)(**9**) was synthesized *via* the cyclization of the corresponding linear tetrapeptide glycyl-[*S*-(*p*-methoxybenzyl)-L-cystyl]sarcosyl-L-prolyl-*p*-nitrophenyl ester hydrochloride (HCl·H–Gly–L-Cys(Bzl(OMe))–Sar–L-Pro–ON_P) (**8**) in a dilute solution of pyridine. Cyclo(Gly–L-Cys–Sar–L-Pro) (**10**) was afforded by the treatment of CGCSP (**9**) with anhydrous hydrogen fluoride (HF). The final product, BCGCSP (**11**), was obtained from **10** by bubbling with oxygen in the presence of 1,2-diiodoethane as the oxidizing agent.[25] The intermediate peptide fragments and the resulting peptide were purified thoroughly by gel filtration and/or crystallization where possible. The identity and purity of the peptides were checked by a combination of thin-layer chromatography (TLC), nuclear magnetic resonance (NMR), and infrared spectroscopy (IR). We used the I solvent system (chloroform : methanol : acetic acid=95 : 5 : 1 by volume), and the II solvent system (chloroform : methanol =5 : 1, by volume) for the TLC. The synthetic procedures are described in detail below.

N-(t-Butoxycarbonyl)sarcosyl-L-proline Methyl Ester (1). A solution of Boc–Sar–OH (13.27 g, 70 mmol) in chloroform (50 ml) was cooled to −20 °C and then treated with *N*-methylmorpholine (7.71 ml, 70 mmol) and isobutyl chloroformate (9.19 ml, 70 mmol) to prepare the mixed anhydride. After 3 min, a solution of HCl·H–L-Pro–OMe (11.62 g, 70 mmol) in chloroform (20 ml), were added to the reaction mixture, which was then stirred at −15 °C for 1 h. It was subsequently allowed to warm to room temperature slowly with continuous stirring. After 24 h, the reaction mixture was diluted with chloroform (30 ml) and then extracted successively with 100 ml of water (2 times), 4% sodium hydrogencarbonate (2 times), and a saturated sodium chloride aqueous solution (2 times). The chloroform layer was separated and dried over anhydrous sodium sulfate overnight. After the removal of the sodium sulfate by filtration, the chloroform was evaporated to yield the linear dipeptide as a pale yellow syrup. Crystallization attempts were unsuccessful. The material was used for the following reaction without further purification,



Scheme 1. Synthetic route of BCGCSP. Abbreviations: Boc; *t*-butoxycarbonyl, Sar; *N*-methylglycine, –OMe; methyl ester, DCHA; dicyclohexylamine, MA; mixed anhydride method, –Bzl(OMe); methoxybenzyl, 3.5 M HCl/EtOAc, 3.5 M anhydrous hydrogen chloride in ethyl acetate, DCC; dicyclohexylcarbodiimide, *p*-N_p; *p*-nitrophenol, –ON_p, *p*-nitrophenyl ester.

since it was judged to be the desired product on the basis of TLC: yield, 21.02 g (100%); $R_f^I=0.41$, $R_f^{II}=0.77$.

Sarcosyl–L-proline Methyl Ester Hydrochloride (2). 3.5 M HCl in ethyl acetate (600 ml) was added to Boc–Sar–L-Pro–OMe (1) (21.02 g, 70 mmol). The reaction mixture was then allowed to stand at room temperature for 1 h. The subsequent removal of the solvent by evaporation left a pale yellow syrup. Trituration with ether yielded a white solid. Recrystallization from methanol–ethyl acetate–ether gave HCl·H–Sar–L-Pro–OMe (**2**) as a white solid: yield, 13.92 g (84%); mp 167—168 °C; $R_f^I=0.03$, $R_f^{II}=0.21$. Found: C, 45.64; H, 7.23; N, 11.81%. Calcd for $C_9H_{17}O_3N_2Cl$: C, 45.67; H, 7.24; N, 11.83%.

N-(t-Butoxycarbonyl) [*S-(p-methoxybenzyl)-L-cysteinyl*] *sarcosyl-L-proline Methyl Ester (3).* Dicyclohexylcarbodiimide (DCC) (1.95 g, 9.45 mmol) in chloroform (10 ml) was stirred into a solution of dicyclohexylammonium salt of Boc–L-Cys(Bzl(OMe))–OH (Boc–L-Cys(Bzl(OMe))–OH·DCHA, 4.94 g, 9.45 mmol) and HCl·H–Sar–L-Pro–OMe (**2**) (2.24 g, 9.45 mmol) in chloroform (60 ml) at −10 °C. The reaction mixture was stirred for 5 h at −5 °C and then overnight at room temperature. After the removal of *N,N'*-dicyclohexylurea, the filtrate was evaporated and the residue was diluted with ethyl acetate (100 ml). The precipitate was filtered off, and the filtrate was successively washed with 100

ml of 10% citric acid (2 times), 4% sodium hydrogencarbonate (3 times), and water (2 times). The organic layer was dried over anhydrous sodium sulfate and concentrated to leave a colorless syrup, which was then triturated with hexane. An attempted recrystallization from ether–hexane was unsuccessful. The Boc-tripeptide (**3**) was obtained as a white semisolid: yield 4.16 g (84%(; $R_f^{I}=0.44$, $R_f^{II}=0.80$; $[\alpha]_D^{25}=-75.9°$ ($c=1.19$). Found: C, 57.41; H, 7.17; N, 7.96%. Calcd for $C_{25}H_{37}O_7N_3S$: C, 57.34; H, 7.12; N, 8.02%.

[S-(p-*Methoxybenzyl*)-L-*cysteinyl*]*sarcosyl*-L-*proline Methyl Ester Hydrochloride* (**4**). This tripeptide hydrochloride was prepared by the removal of the Boc group of tripeptide Boc–L-Cys(Bzl(OMe))–Sar–L-Pro–OMe (**3**) (4.00 g, 7.64 mmol), as has been described above for HCl·H–Sar–L-Pro–OMe (**2**). Recrystallization from ethanol–ether gave a white solid: yield 3.51 g (100%); mp 83—84 °C; $R_f^{I}=0.03$, $R_f^{II}=0.54$. Found: C, 52.18; H, 6.64; N, 9.11%. Calcd for $C_{20}H_{30}O_5N_5SCl$: C, 52.22; H, 6.57; N, 9.13%.

N-(t-*Butoxycarbonyl*)*glycyl*-[S-(p-*methoxybenzyl*)-L-*cysteinyl*]-*sarcosyl*-L-*proline* (**6**). Boc–Gly–OH·DCHA (2.56 g, 7.17 mmol) was coupled with HCl·H–L-Cys(Bzl(OMe))–Sar–L-Pro–OMe (**4**) (3.30 g, 7.17 mmol), using DCC (1.48 g, 7.17 mmol) in chloroform (100 ml) in the same manner as that used for the preparation of Boc–L-Cys(Bzl(OMe))–Sar–L-Pro–OMe (**3**). Recrystallization from ether–hexane gave Boc–Gly–L-Cys(Bzl(OMe))–Sar–L-Pro–OMe (**5**) as a pale yellow semisolid: yield 3.04 g (73%); mp 49—50 °C; $R_f^{I}=0.27$, $R_f^{II}=0.76$; $[\alpha]_D^{25}=-84.8°$ ($c=1.00$).

Boc–Gly–L-Cys(Bzl(OMe))–Sar–L-Pro–OMe (**5**) (3.00 g, 5.17 mmol) in methanol (25 ml) was treated with 1 M NaOH (10.33 ml, 10.33 mmol). After 1.5 h, the methanol was evaporated and the residual solution was diluted with water (25 ml). The aqueous solution was washed with ether (30 ml) and acidified with 10% citric acid under cooling. The resulting precipitate was extracted with ethyl acetate (100 ml), and the organic layer was dried over anhydrous sodium sulfate. After the removal of the sodium sulfate, the solvent was evaporated to leave a colorless foam, which was solidified by the addition of petroleum ether. The product was recrystallized from ethyl acetate–petroleum ether: yield, 2.93 g (89%). mp 82—84 °C; $R_f^{I}=0.06$, $R_f^{II}=0.17$. Found: C, 54.66; H, 6.80; N, 9.31%. Calcd for $C_{26}H_{38}O_8N_4S\cdot 0.5H_2O$: C, 54.25; H, 6.83; N, 9.73%.

N-(t-*Butoxycarbonyl*)*glycyl*-[S-(p-*methoxybenzyl*)-L-*cysteinyl*]-*sarcosyl*-L-*proline* p-*Nitrophenyl Ester* (**7**). Into a solution of Boc–Gly–L-Cys(Bzl(OMe))–Sar–L-Pro–OH (**6**) (1.60 g, 2.82 mmol) and *p*-nitrophenol (0.43 g, 3.10 mmol, pulverized previously) in chloroform (15 ml), we stirred a solution of DCC (0.64 g, 3.10 mmol) in chloroform (3 ml) at −5 °C. Stirring was continued at 0 °C overnight. Monitoring the reaction mixture by TLC showed a small amount of the unreacted starting material, so that 0.078 g more of *p*-nitrophenol and 0.058 g of DCC were added after 20 h. Additional stirring was continued at 5 °C for 5 h. The solution was then filtered to remove the precipitated urea, treated with 3 drops of acetic acid, and stirred for 1 h. The solution was subsequently filtered again to ensure the complete removal of the additional amount of urea. The evaporation of the solvent left a pale brown foam, which was triturated with ether–hexane and dried *in vacuo*. The Boc-tetrapeptide *p*-nitrophenyl ester (**7**) was obtained as an off-white solid: yield 1.80 g (92.8%); mp 59—60 °C; $R_f^{I}=0.07$, $R_f^{II}=0.76$; $[\alpha]_D^{25}=-78.7°$ ($c=1.00$). Found: C, 56.18; H, 5.98; N, 9.92%. Calcd for $C_{32}H_{41}O_{10}N_5S$: C, 55.88; H, 6.01; N, 10.18%.

Cyclo[*glycyl*-[S-(p-*methoxybenzyl*)-L-*cysteinyl*]*sarcosyl*-L-*prolyl*] (**9**). A solution of HCl·H–Gly–L-Cys(Bzl(OMe))–Sar–L-Pro–ON_p (**8**) (1.61 g, 2.58 mmol) (prepared by the treatment of Boc–Gly–L-Cys(Bzl(OMe))–Sar–L-Pro–ON_P (**7**) with 3.5 M HCl in ethyl acetate at 0 °C for 50 min, trituration with ether, and drying *in vacuo*) in *N,N*-dimethylformamide (DMF) 10 ml) (distilled over calcium hydride just before use), containing a few drops of glacial acetic acid, was added drop by drop and over a period of 5 h to a stirred spectral-grade pyridine (860 ml) at 60 °C. The stirring of the reaction mixture was continued at 30 °C overnight and then allowed to proceed for 3 h at 60 °C. The complete evaporation of pyridine and DMF under reduced pressure left an oily brown residue which was subsequently taken up by water–methanol (1 : 3 by volume) (20 ml). The insoluble material was filtered off, and the filtrate was eluted through columns (1.7 × 17 cm) packed with ion-exchange resin of IR-120B (H^+-type) and IRA-400 (OH^--type) successively. The columns were washed with the same solvent until the color of the eluate disappeared. The combined effluent (*ca.* 300 ml) was concentrated to yield a pale yellow syrup. Trituration with ether gave an off-white solid. The crude product (100 mg) was dissolved in a minimum amount of methanol and chromatographed on a Sephadex LH-20 column (2.7 × 85 cm), using methanol as the eluate. A total of 7 ml × 60 fractions were collected. The single components (Nos. 40—46 tubes) were collected, and the solvent was evaporated. The residual syrup was triturated with ether. Recrystallization from methanol–water–ether gave a white solid of cyclo[Gly–L-Cys(Bzl(OMe))–Sar–L-Pro] (**9**); yield 90 mg; ninhydrin test, negative; IR (KBr disk); 3450 (NH str.), 2950 (CH str.), 1650 cm^{-1} (C=O str,); mp 142—143 °C; $R_f^{I}=0$, $R_f^{II}=0.47$; $[\alpha]^{25}=-79.2°$ ($c=0.59$). Found: $m/e=448$. Calcd for $C_{21}H_{28}O_5N_4S$: M= 448. Found: C, 55.17; H, 6.12; N, 12.12%. Calcd for $C_{21}H_{28}O_5N_4S\cdot 0.5H_2O$: C, 55.13; H, 6.39; N, 12.25%.

S,S′-*Bis*[*cyclo*(*glycyl*-L-*hemicystyl*-*sarcosyl*-L-*prolyl*) (**11**). Cyclo[Gly–L-Cys(Bzl(OMe))–Sar–L-Pro] (**9**) (90 mg, 0.2 mmol) was placed in an HF-reaction reservoir vessel together with anisole (0.3 ml). Anhydrous HF was added the reservoir vessel using an HF-reaction apparatus, after which the mixture was allowed to react at 0 °C for 1 h while being stirred. The excess HF was removed under reduced pressure at 0 °C, and the residue was dried under reduced pressure for 6 h at room temperature. Subsequent crystallization from methanol–ether gave an off-white solid as cyclo(Gly–L-Cys–Sar–L-Pro) (**10**). The material was immediately used in the next step without further purification. 1,2-diiodoethane (0.042 g, 0.15 mmol) (crystallized from ethanol just before use) and triethylamine (0.056 ml, 0.4 mmol) were stirred into a solution of cyclo(Gly–L-Cys–Sar–L-Pro) (**10**) in methanol (15 ml) under hydrogen.[23] The reaction mixture was then stirred at room temperature overnight. Monitoring the reaction mixture by a nitroprusside test showed it still positive, so an additional amount of 1,2-diiodoethane (0.020 g) was added, after which air was bubbled through the reaction mixture for 6 h. The methanol-insoluble white powdery material was separated on standing overnight: it was then filtered off and thoroughly washed with methanol. The product was found to be insoluble in most organic solvents, such as acetone, chloroform, and ethanol. Recrystallization from water–methanol gave an off-white solid of bis(cyclic tetrapeptide) (**11**): yield 25 mg (38%); ninhydrin test, negative; nitroprusside test, negative; IR (KBr disc) 3450 (NH str.), 2920 (CH str.), 1640 cm^{-1} (C=O str.); mp 280—285 °C (decomp.); $R_f^{I}=0$, $R_f^{II}=0.11$. Found: C, 44.26; H, 5.75; N, 15.61%. Calcd for $C_{26}H_{38}O_8N_8S_2\cdot 3H_2O$: C, 44.06; H, 6.26; N, 15.81%.

Procedures. All the melting points were measured on a Yanagimoto Melting-point Apparatus and were not corrected. The optical rotations were measured with a Perkin-Elmer 241 polarimeter. The IR spectra were taken on a

Shimadzu spectrometer, model IR-430.

The ^{1}H-NMR spectra obtained at 360.06 MHz on a NICOLET NT-360 spectrometer with the NIC 1180 Computer Data System, operating in the Fourier transform mode with quadrature detection. The chemical shifts are reported in parts per million (ppm) downfield from internal tetramethylsilane (TMS). The ^{13}C-NMR data were recorded on either a NICOLET NT-360 or a Varian CFT-20 spectrometer at 90.54 MHz or 20.12 MHz respectively, in the Fourier transform mode with proton-noise decoupling. Chemical shifts are given in ppm relative to the internal TMS. The Raman spectra of the bis(peptide) in the liquid state were measured with a JEOL Raman 400D spectromer equipped with an argon-ion laser.

The circular dichroism (CD) spectra were recorded on a JASCO J-40A automatic recording spectropolarimeter equipped with a J-DPY data processor at 25 °C. Generally measurements were carried out in quartz cells with a 0.1-cm path length over 250 nm to 200 nm and a 1-cm path length over 350 nm to 250 nm. Data are represented as molar ellipticities. Spectrophotometric-grade methanol and water were used as solvents.

The authors wish to express their thanks to Dr. Yukio Imanishi of Kyoto University for his valuable suggestions and discussions. We also would like to thank Dr. Shoei Fujishige of our Institute for his useful comments.

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