Studies of Peptide Antibiotics. XXXVIII.[1] Synthesis of *S,S'*-Bi([1-L-hemicystine]-gramicidin S), a Dimerized Analog of Gramicidin S

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(Received November 12, 1977)

To investigate the importance of the orientation of side chains of amino acid residues in gramicidin S (GS) for the antibacterial activity, a dimerized analog of GS, namely *S,S'*-bi([1-L-hemicystine]-GS) (**13**) was synthesized *via* removal of *p*-methoxybenzyl group of [1-*S*-*p*-methoxybenzyl-L-cysteine]-GS (**12**) and subsequent oxidation. Compound **12** showed the same antibacterial activity as GS, whereas **13** was inactive. The resemblance of ORD curves of the two analogs to that of GS suggested the similarity of peptide-backbone structure of these three compounds. Strong activity of **12** was explained by similar conformation of **12** to that of GS. To explain the inactivity of **13**, a molecular model was proposed; four hydrophilic side chains are located on surface of the molecule, whereas six hydrophobic are burried.

Gramicidin S (GS) is a cyclic decapeptide antibiotic possessing the amino acid sequence shown in Fig. 1. Several models have been proposed[2] for the conformation of GS in solid state and solution. The most favorable model is the intramolecular antiparallel β-form with four hydrogen bondings between the valyl and the leucyl residues.[3] A characteristic feature of this conformation is the orientation of side chains in which the charged Orn side chains were on one side and the hydrophobic Val and Leu side chains on the other side of the molecule.[4] Recently, Kato and Izumiya introduced the sidedness hypothesis which means that the sidedness in GS molecule is important for the antibacterial activity of GS.[5,6] The sidedness hypothesis are supported by the results from many investigations of synthetic amino acid-substituted analogs.[2]

Fig. 1. Structure of GS and its analogs.

[3,3'-Di-*N*-methyl-L-leucine]-gramicidin S synthesized by Sugano *et al.*[7] showed the same antibacterial activity as GS. For the conformation of this analog, Kumar *et al.*[8] proposed a model, in which the side chains of Orn and Val were on the same side of the molecule, on the basis of NMR studies. On the other hand, Kato and Izumiya[5] showed the possibility of other two models which satisfy the sidedness hypothesis; the models were derived from the calculation of dihedral angles and other considerations. Therefore, it seemed interesting to synthesize an analog of GS which possesses a deviated conformation from the sidedness hypothesis. For this purpose, we selected a dimerized analog of GS containing a disulfide bridge. In this connection, it would be noteworthy that a synthetic digramicidin S, *cyclo*(–Val–Orn–Leu–D-Phe–Pro–)$_4$, possessed an appreciable antibacterial activity, and showed similar ORD curves as GS in ethanol, but quite different in aqueous urea.[9] These results suggested that digramicidin S also possesses somewhat GS-like orientation of side chains in spite of its soft and fairly different structures from that of GS.

The present paper deals with the synthesis, antibacterial properties, and ORD measurements of a dimerized analog of GS, *S,S'*-bi([1-L-hemicystine]-gramicidin S) (**13**), and a precursor of **13**, [1-*S*-*p*-methoxybenzyl-L-cysteine]-gramicidin S (**12**), the structure of them being shown in Fig. 1. The former compound, **13**, was a model in which the hydrophobic sides of two GS molecules were combined together by a disulfide bridge.

The synthesis of **12** was outlined in Fig. 2.[10] Cyclization reaction was achieved with leucyl residue at *C*-terminal.[11] Mercapto group of cysteine was protected with *p*-methoxybenzyl group which was removable smoothly with liquid hydrogen fluoride,[12] but stable for the treatment with hydrogen bromide in acetic acid. Mixed anhydride method was used for couplings between an amino acid and a peptide derivative, and azide method for fragment coupling and cyclization.

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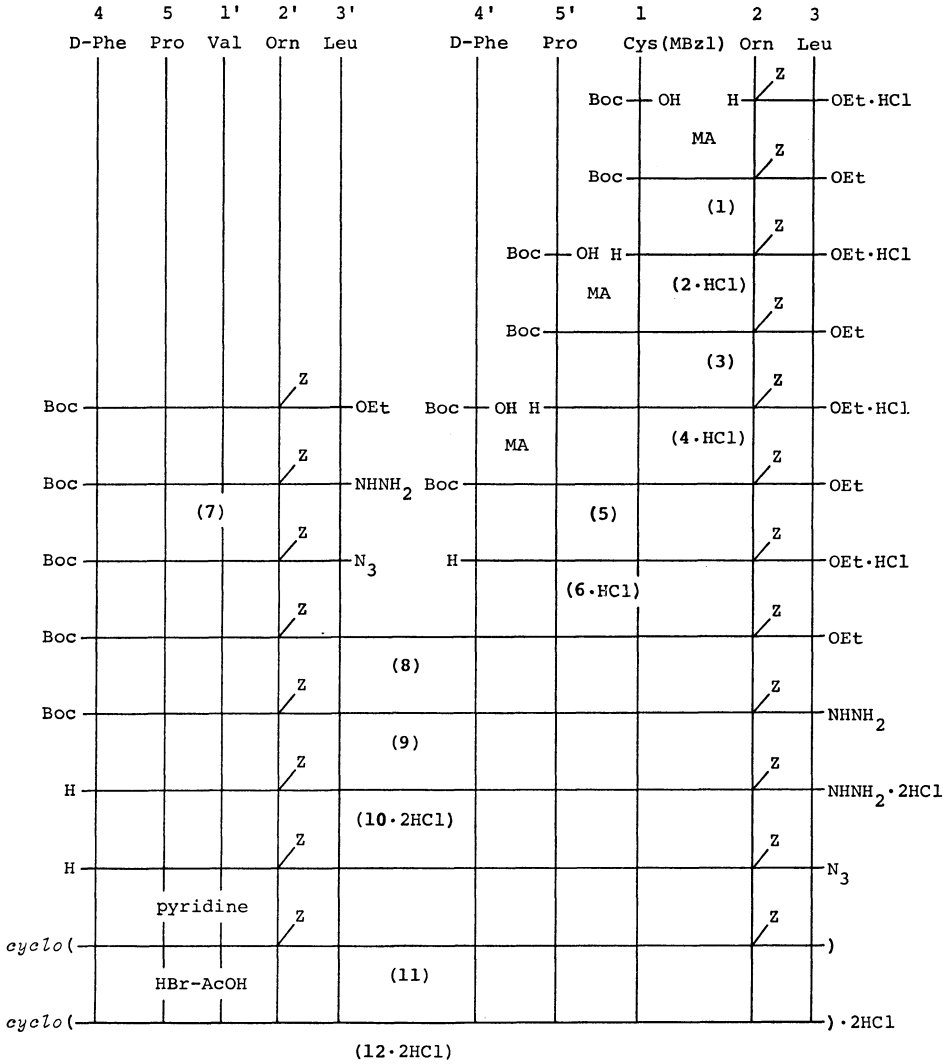

Fig. 2. Synthesis of [Cys(MBzl)[1]]-GS (**12**).

The pentapeptide ester (**6**) containing *S-p*-methoxybenzylcysteine residue was prepared by stepwise elongation from H–Orn(Z)–Leu–OEt·HCl[13] toward the amino end. Azide coupling of **6** and Boc-pentapeptide hydrazide (**7**) derived from Boc–D-Phe–Pro–Val–Orn(Z)–Leu–OEt[13] afforded a Boc-decapeptide ester (**8**), which was purified with silica gel column chromatography. Conversion of **8** to its hydrazide (**9**) and deblocking of Boc-group of **9** were performed without any difficulty. A key interemediate (**11**) was derived by the cyclization of **10** in a satisfactory yield of 64%. Z-groups of **11** were removed with hydrogen bromide in acetic acid and the dihydrobromide (**12**·2HBr) obtained was treated with Dowex 1 (OH^- form) to afford an acid-free **12**, which was converted to a dihydrochloride in a crystalline fine needle like GS dihydrochloride by the addition of ethanol and 1 M hydrochloric acid. The desired compound, **13**, was derived from **12** by the treatment with liquid hydrogen fluoride followed by oxidation with potassium hexacyanoferrate(III).[14] The molecular size of **13** shown in Fig. 1 was estimated by the fact that the elution volume of **13**·4HCl in the column chromatography of Sephadex G-25 coincided well with that of digramicidin S tetrahydrochloride.[9] The homogeneity of **12** and **13** was confirmed by paper and thin-layer chromatography, paper electrophoresis and elemental analysis.

Table 1. Antibacterial activity of GS and its analogs (Minimum inhibitory concentration, μg/ml)

Strain	GS	[Cys(MBzl)[1]]-GS (**12**)	*S,S'*-Bi([hemicystine[1]]-GS) (**13**)
Staphylococcus aureus	5	10	>100
Bacillus subtilis	2	5	>100
Escherichia coli	>100	>100	>100
Mycobacterium avium	100	>100	>100
Proteus vulgaris	>100	>100	>100

Antibacterial activities of **12** and **13** measured in nutrient agar are listed in Table 1. Compound **12** exhibited the same activity as GS against some organisms tested, whereas **13** was inactive. These data indicated that the Val[1] residue in GS was exchangeable to Cys(MBzl) residue without any significant effect on the antibacterial activity, and that the activity of **12**

was lost when the hydrophobic sides in position 1 were combined with a disulfide bridge.

The ORD curves of the two analogs as well as of GS are shown in Fig. 3. The fact that **12** exhibited closely similar curves to GS in EtOH (Fig. 3-a) and even in the strong denaturing agent such as 8 M urea (Fig. 3-b) suggested the rigid and stable conformation of **12** as well as natural GS. The dimerized analog, **13**, also exhibited the same pattern as GS though the negative trough was shallower than that of GS, the result suggesting that the structure of peptide-backbone was not changed significantly by the formation of a disulfide bridge.

The results of biological assay and ORD measurement

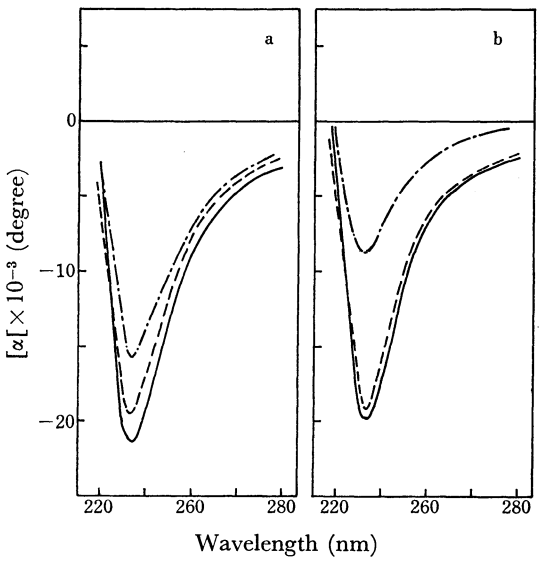

Fig. 3. ORD curves of GS and its analogs. Solvent: a, EtOH; b, 8 M urea. Curve: ——, GS; – – –, **12**; -·-·-, **13**.

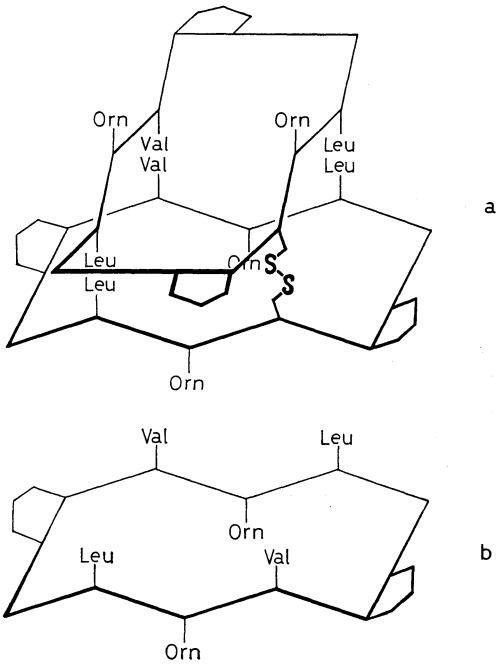

Fig. 4. A possible conformation (a) of a dimerized analog (**13**) and a conformation (b) of GS.

of **12** indicated the importance of definite orientation of side chains such that the hydrophilic side chains were on one side of a molecule and the hydrophobic were on the other side for the molecule such as GS and its active analogs. On the other hand, we propose a possible conformation (Fig. 4-a) of **13** suitable for the results of its biological assay and ORD measurement. In this model, (1) the H^{α}–C^{α} and C^{β}–S bonds are in antiperiplanar conformation regards to the C^{α}–C^{β} bond,[15] (2) the dihedral angle of C^{β}–S–S′–C^{β} is +90° (P-helical), and (3) charged side chains of four Orn residues are located on surface of the molecule, and bulky side chains of six Val and Leu residues make three pair of hydrophobic interactions in inside. In this connection, it would be noteworthy that NMR and CD studies on a bicyclic GS analog (cyclic decapeptide), [2,2′-cystine]-GS (**14**), revealed that its structure was identical with that of GS;[16] the fact indicated no distorting of the conformation of **14** by disulfide bridge which links the two β-antiparallel strands. The biological activity of **14**, however, has not been reported.

Experimental

All the melting points are uncorrected. The ratio in parentheses after a solvent system was indicated by vol. TLC was carried out on silica gel G (Merck) with the following solvent systems: R_f^1, CHCl–MeOH (5:1); R_f^2, *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2). Paper chromatography was carried out on Toyo Roshi No. 52 paper; R_f^3, same solvent as that used for R_f^2. Optical rotations were measured on a Union high sensitivity polarimeter PM-71. Amino acid analyses were performed with a Hitachi amino acid analyzer KLA-5. Molecular weight was determined with a Hitachi Osmometer, Model 115, MeOH being used as a solvent.

Boc-Cys(MBzl)-Orn(Z)-Leu-OEt (1). To a chilled solution of Boc-Cys(MBzl)-OH (5.12 g, 15 mmol) and TEA (2.1 ml, 15 mmol) in THF (30 ml) was added isobutyl chloroformate (1.97 ml, 15 mmol) at −10°C. After 10 min, a chilled solution of H-Orn(Z)-Leu-OEt·HCl[13] (6.65 g, 15 mmol) and TEA (2.1 ml, 15 mmol) in $CHCl_3$ (30 ml) was added. The mixture was left to stand at room temperature overnight, evaporated *in vacuo*, and the oily residue was dissolved in EtOAc. The solution was washed successively with 4% $NaHCO_3$, 10% citric acid and water, dried (Na_2SO_4), and evaporated under reduced pressure. The residual oil was solidified by the addition of ether and petroleum ether, and the product was recrystallized from EtOH–ether–petroleum ether; yield, 7.68 g (70%); mp 78—80 °C; $[\alpha]_D^{20}$ −25.6° (*c* 1, EtOH); R_f^1 0.88.

Found: C, 60.90; H, 7.50; N, 7.77%. Calcd for $C_{37}H_{54}O^9N_4S$: C, 60.80; H, 7.45; N, 7.67%.

H-Cys(MBzl)-Orn(Z)-Leu-OEt·HCl (2·HCl). Compound **1** (6.58 g, 9 mmol) was dissolved in 2.8 M hydrogen chloride in EtOAc (64 ml). The solution was allowed to stand at room temperature for 1 h and then evaporated. Addition of ether to the oily residue gave hygroscopic crystals; yield, 5.47 g (91%); mp 170—171 °C; $[\alpha]_D^{20}$ −10.6° (*c* 1, EtOH); R_f^1 0.80, R_f^2 0.84, R_f^3 0.93.

Found: C, 56.42; H, 7.08; N, 8.44%. Calcd for $C_{32}H_{46}O_7N_4S\cdot HCl\cdot H_2O$: C, 56.08; H, 7.21; N, 8.18%.

Boc-Pro-Cys(MBzl)-Orn(Z)-Leu-OEt(3). This was prepared from Boc-Pro-OH (1.64 g, 7.6 mmol) and **2**·HCl (5.09 g, 7.6 mmol) as described for the preparation of **1**.

The product was recrystallized from EtOH–ether; yield, 5.16 g (82%); mp 134—136 °C; $[\alpha]_D^{20}$—48.8° (*c* 1, EtOH); R_f^1 0.87.

Found: C, 60.44; H, 7.52; N, 8.39%. Calcd for $C_{42}H_{61}O_{10}N_5S$: C, 60.92; H, 7.43; N, 8.46%.

H–Pro–Cys(MBzl)–Orn(Z)–Leu–OEt·HCl (**4**·*HCl*). This was prepared from **3** (2.85 g, 3.5 mmol) as described for **2**·HCl; yield, 2.52 g (95%); mp 112—114 °C; $[\alpha]_D^{20}$—39.0° (*c* 1, EtOH); R_f^1 0.66, R_f^2 0.78.

Found: C, 57.37; H, 7.13; N, 8.87%. Calcd for $C_{37}H_{53}O_8N_4S$·HCl: C, 57.46; H, 7.17; N, 9.06%.

Boc–D-Phe–Pro–Cys(MBzl)–Orn(Z)–Leu–OEt (**5**). This was prepared from Boc–D-Phe–OH (0.85 g, 3.2 mmol) and **4**·HCl (2.47 g, 3.2 mmol) as described for **1**. The product was obtained as a foam; yield, 3.01 g (97%); $[\alpha]_D^{20}$—85.9° (*c* 2, EtOH); R_f^1 0.71.

Found: C, 62.66; H, 7.44; N, 8.50%. Calcd for $C_{51}H_{70}O_{11}N_6S$: C, 62.81; H, 7.24; N, 8.62%.

H–D-Phe–Pro–Cys(MBzl)–Orn(Z)–Leu–OEt·HCl (**6**·*HCl*). Compound **5** (1.95 g, 2 mmol) was treated with 2.8 M hydrogen chloride in EtOAc (14 ml) as described for **2**·HCl. The oily product was purified with a column (4.6×40 cm) of silica gel as follows. The crude **6**·HCl was applied on the column. The column was washed successively with EtOAc (1000 ml) and a mixture (300 ml) of EtOAc–EtOH (2:1), and eluted with EtOH (700 ml). Each fraction was assayed by TLC, and the fractions containing the desired product were evapotared to leave a foam; yfield, 1.29 g (70%); $[\alpha]_D^{20}$—119° (*c* 1, EtOH); R_f^1 0.62.

Found: C, 59.83; H, 7.00; N, 9.11%. Calcd for $C_{46}H_{62}O_9N_6S$·HCl·1/2H_2O: C, 60.01; H, 7.01; N, 9.13%.

Boc–D-Phe–Pro–Val–Orn(Z)–Leu–N_2H_3 (**7**). A solution of Boc–D-Phe–Pro–Val–Orn(Z)–Leu–OEt (0.86 g, 1 mmol)[13] and hydrazine hydrate (1.94 ml, 40 mmol) in DMF (6 ml) was allowed to stand at room temperature for 48 h. The solution was evaporated and the addition of water afforded a solid, which was recrystallized from MeOH–ether; yield, 0.77 g (92%); mp 178—182 °C; $[\alpha]_D^{20}$—62.8° (*c* 1, EtOH); R_f^1 0.72.

Found: C, 61.43; H, 7.95; N, 13.23%. Calcd for $C_{43}H_{64}O_9N_8$: C, 61.70; H, 7.71; N, 13.39%.

Boc–D-Phe–Pro–Val–Orn(Z)–Leu–D-Phe–Pro–Cys(MBzl)–Orn(Z)–Leu–OEt (**8**). To a solurion of **7** (313 mg, 0.37 mmol) in DMF (2 ml) were added 2.8 M hydrogen chloride in EtOAc (0.52 ml) and isopentyl nitrite (0.057 ml, 0.41 mmol) in DMF (0.57 ml) at −60 °C. After being left to stand at −20 °C for 10 min, the solution was cooled again to −60 °C and neurtalized with TEA (0.21 ml, 1.5 mmol). To this solution was added a chilled solution of **6**·HCl (337 mg, 0.37 mmol) and TEA (0.05 ml, 0.37 mmol) in DMF (1 ml). The reaction mixture was allowed to stir at 0 °C for 48 h and then evaporated. After the addition of 0.02 M citric acid (100 ml), the solid was collected and washed with water. The crude product was purified with a column (4.6×45 cm) of silica gel using $CHCl_3$–MeOH–AcOH (95:5:1) as a developing agent as described for **6**·HCl; yield, 370 mg (60%); mp 104—109 °C; $[\alpha]_D^{20}$—116° (*c* 0.5, EtOH); R_f^1 0.79.

Found: C, 63.32; H, 7.31; N, 9.96%. Calcd for $C_{89}H_{122}O_{18}N_{12}S$·$H_2O$: C, 63.32; H, 7.40; N, 9.95%.

Boc–D-Phe–Pro–Val–Orn(Z)–Leu–D-Phe–Pro–Cys(MBzl)–Orn(Z)–Leu–N_2H_3 (**9**). Compound **8** (336 mg, 0.2 mmol) was treated with hydrazine hydrate (0.39 ml, 8 mmol) as described for **7**; yield, 232 mg (70%); mp 208—209 °C $[\alpha]_D^{20}$—126° (*c* 0.5, EtOH); R_f^1 0.61.

Found: C, 61.12; H, 7.19; N, 11.55%. Calcd for $C_{87}H_{120}O_{17}N_{14}S$·$2H_2O$: C, 61.39; H, 7.34; N, 11.52%.

H–D-Phe–Pro–Val–Orn(Z)–Leu–D-Phe–Pro–Cys(MBzl)–Orn(Z)–Leu–N_2H_3·2*HCl* (**10**·2*HCl*). Compound **9** (216 mg, 0.13 mmol) was treated with 0.1 M hydrogen chloride in formic acid (3.3 ml) as described for **2**·HCl and the product was used for the next reaction without further treatment; yield, 196 mg (92%); R_f^1 0.58.

cyclo(*–D-Phe–Pro–Val–Orn(Z)–Leu–D-Phe–Pro–Cys(MBzl)–Orn(Z)–Leu–*) (**11**). To a solution of **10**·2HCl (178 mg, 0.11 mmol) in DMF (2 ml) were added 2.8 M hydrogen chloride in EtOAc (0.15 ml) and isopentyl nitrite (0.017 ml, 0.12 mmol) in DMF (0.17 ml) at −30 °C. After 30 min, the reaction mixture was added into pyridine (108 ml) at 0 °C. After being stirred at 5 °C for 4 days, the solution was evaporated and the residue was dissolved in a mixture (11 ml) of MeOH–H_2O (10:1). The solution was applied on columns (1.9×20 cm each) of Dowex 1 (OH^- form) and Dowex 50 (H^+ form). The columns were washed with the same solvent (200 ml); the combined effluent was evaporated to leave an oil which was solidified by the addition of water. The crude product dissolved in MeOH (2 ml) was applied on a column (1.8×110 cm)of Sephadex LH-20 which was developed with MeOH. The fractions with the desired product were evaporated and the oily residue was crystallized by the addition of ether and petroleum ether; yield, 106 mg (64%); mp 187—189 °C; $[\alpha]_D^{20}$—290° (*c* 0.5, MeOH); R_f^1 0.78.

Found: C, 63.54; H, 7.05; N, 10.71%; mol wt, 1541. Calcd for $C_{82}H_{108}O_{15}N_{12}S$·$H_2O$: C, 63.46; H, 7.15; N, 10.83%; mol wt, 1552.

cyclo(*–D-Phe–Pro–Val–Orn–Leu–D-Phe–Pro–Cys(MBzl)–Orn Leu–*)·2*HCl* ([*Cys(MBzl)*[1]]–*GS*·2*HCl*) (**12**·2*HCl*). To a solution of **11** (96 mg, 0.06 mmol) in AcOH (1 ml) was added 25% hydrogen bromide in AcOH (0.2 ml). The rereaction mixture was allowed to stand at room temperature for 4 h, evaporated, and the yellowish residue was crystallized by the addition of ether. The crude dihydrobromide was dissolved in a mixture of MeOH–H_2O (4:1) and the solution was applied on a column (1.9×20 cm) of Dowex 1 (OH^- form). The peptide in the column was eluted with the same solvent and the combined effluent (200 ml) was evaporated to leave a solid. The product was recrystallized from EtOH–1 M HCl; yield, 62 mg (75%); mp 270—271 °C (dec); $[\alpha]_D^{20}$—297° (*c* 0.3, EtOH); R_f^2 0.84, R_f^3 0.80.

Found: C, 55.64; H, 7.59; N, 11.45%. Calcd for $C_{66}H_{96}O_{11}N_{12}S$·2HCl·$5H_2O$: C, 55.49; H, 7.61: N, 11.75%.

S,S'-Bicyclo(*–D-Phe–Pro–Val–Orn–Leu–D-Phe–Pro–hemicystyl–Orn–Leu–*)·4*HCl* (S,S'–*Bi*([*hemicystine*[1]]–*GS*)·4*HCl*) (**13**·4*HCl*). To a mixture of **12**·2HCl (40 mg, 0.03 mmol) and anisole (0.16 ml) was added liquid hydrogen fluoride (5 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and then evaporated *in vacuo*. The residue was dissolved in 1 M AcOH (50 ml) and the solution was washed with ether (20 ml×2) and then lyophilized. To a solution of the lyophilizate (30 mg) in 0.05 M ammonium acetate containing 30% MeOH (50 ml, pH 6.8) was added 0.01 M potassium hexacyanoferrate(III) (5.6 ml).[14] After being stirred for 10 min, Amberlite IR-45 (Cl^- form) resin was added into the solution, and the resin was filtered off and washed with 30% MeOH. The filtrate and washings were evaporated, the residue was dissolved in a mixture (3 ml) of AcOH–MeOH–H_2O (1:1:1), and the solution was applied on a column of Sephadex G-25 (2×80 cm) which was eluted with the same solvent. The fractions of the desired product were evaporated to leave a solid, which was recrystallized from EtOH–1 M HCl; yield, 15 mg (41%); mp 258—259 °C (dec); $[\alpha]_D^{20}$—287° (*c* 0.4, EtOH); R_f^2 0.75; R_f^3 0.80. Amino acid ratios in acid hydrolyzate; Phe

4.20, Pro 3.76, Val 1.92, cystine 0.90, Orn 4.12, Leu 4.00. Found: C, 53.43; H, 7.37; N, 12.07%. Calcd for $C_{116}H_{174}O_{20}N_{24}S_2 \cdot 4HCl \cdot 10H_2O$: C, 53.28; H, 7.63; N, 12.36%.

Paper Electrophoresis. This was carried out with Toyo Roshi No. 52 paper and a solvent system of HCOOH–AcOH–MeOH–H_2O (1: 3: 6: 10, pH 1.8) for 2.5 h at 500 V/30 cm. Figure 5 showed that each of the peptides (**12** and **13**) revealed a single spot.

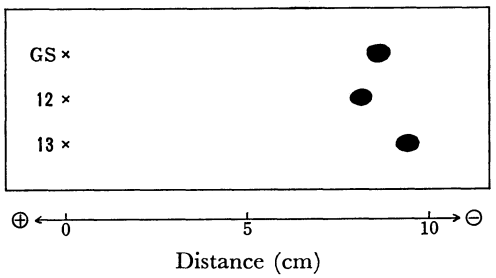

Fig. 5. Paper electrophoresis of GS and its analogs.

Microbiological Assays[17] *and ORD Measurements.* These were carried out as described in a previous paper,[13] the results being shown in Table 1 and Fig. 3.

The authors wish to express their thanks to Dr. T. Kato and Dr. M. Waki of this laboratory for their helpful discussions.

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6) In 1958, Schwyzer proposed the concept that GS shows the antibacterial activity because of a specific conformation of the molecule in which the charged side chains are on one side and the hydrophobic side chains on the other side.[4] For this concept, Kato and Izumiya introduced a term of sidedness hypothesis in 1977;[5] a term of sidedness itself appeared at first in 1975.[8]

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10) Abbreviations according to IUPAC-IUB Commission, *J. Biol. Chem.*, **247**, 977 (1972), are used throughout. Additional abbreviations: DMF, *N*,*N*-dimethylformamide; MA, mixed anhydride; MBzl, *p*-methoxybenzyl; TEA, triethylamine. Amino acid symbols except D-Phe denote the L-configuration.

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17) We are indebted to the staff of Takeda Chemical Industries, Ltd., for the assay.