ACYLATION OF ORNITHINE RESIDUES IN GRAMICIDIN S WITH DIAMINOMONOCARBOXYLIC ACIDS

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ACYLATION OF ORNITHINE RESIDUES IN GRAMICIDIN S WITH DIAMINOMONOCARBOXYLIC ACIDS

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Abstract

Gramicidin S analogs which acylated with diaminomonocarboxylic acids were synthesized. The antibacterial activities of these analogs were equal to or weaker than that of gramicidin S. Adsorption of analogs on bacterial cells and the conformation of the analogs were discussed.

Introduction

Gramicidin S (GS) is an antibiotic with a cyclic decapeptide structure, cyclo (-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-)(*1*). The conformation of GS was established by X-ray analysis as a β-pleated sheet structure (*2*). In this conformation, four hydrophobic side chains of valyl and leucyl residues are located on one side of a ring frame consisting of a cyclic peptide chain, while two hydrophilic side chains of ornithyl residues are located on the opposite side. Accordingly, GS seems to be a cationic surface active agent. Many analogs of GS have been synthesized to investigate the relationship between chemical structure and biological activity. Particularly, ornithine modified analogs of GS have been prepared. Analogs containing no positive charge are inactive, and analogs containing one positive charge are only 1/4-1/10 as active to that of GS (*3,4*). While analogs containing two positive charges have same activity to that of GS (*5,6*). In this manner, antibacterial activity of GS analogs increase with increasing in the number of postive charges. Consequently, analogs containing much positive charges than GS may have stronger activity than that of GS. However, when positive charges are introduced in positions 1,3,4 or 5, the structure of cationic surface active agent of GS destroies.

On the other hand, Stepanov *et al.* reported that, [Orn $(Gly)^{2,2'}$]-GS had high activity (*7*). Acylation of δ-amino group of ornithine with monoaminomonocarboxylic acids forms ornithine analogs with same basic character but have longer side chain. Acylated GS has a

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Abbreviations : GS, gramicidin S; Dap, diaminopropionic acid; Dab, diaminobutylic acid; Amino acid symbols denote the L configulation; DCCC, Droplet countercurrent chromatography.

conformation similar to that of GS. Accordingly, acylation of GS with diaminomonocarboxylic acids gives GS analogs containing much positive charges than GS, and antibacterial activity of these analogs may be stronger than that of GS.

In this experiment, we synthesized GS analogs acylated with monoaminomonocarboxylic acid (Ala, Ser), diaminomonocarboxylic acid (Dap, Dab, Orn, Lys) and monoaminodicarboxylic acid (Asp).

Experimental Procedures

Z-Dap(Z)-OH, Z-Dab(Z)-OH, Z-Orn(Z)-OH, Z-Lys(Z)-OH, Z-Ala-OH, Z-Asp(OBzl)-OH were prepared in the usual way. Boc-Ser(Bzl)-OH was purchased from Protein Research Foundation, Osaka, Japan. ^{14}C-Labeled GS (282 dpm/1μg) was prepared according to the previous paper (*8*). For amino acid analysis, peptides were hydrolyzed with 6 M HCl at 110°C for 24 hr and analyzed on Hitachi amino acid analyzer KLA-5. Droplet countercurrent chromatography (DCCC) was carried out with a chromatographic apparatus made by Seikagaku Kogyo Co., Tokyo, Japan. ORD measurements were performed with a JASCO spectropolarimeter J-20A. Radioactivity was measured with Beckman LS-3150T liquid scintillation counter.

Syntheses of Protected Amino Acid p-Nitrophenylesters —— The synthesis of Z-Dap(Z)-ONp is described as an example. To a solution of Z-Dap(Z)-OH (0.78 g, 2 mmol) and *p*-nitrophenol (0.34 g, 2.4 mmol) in CH_2Cl_2 (10 ml), DCC (0.45 g, 2.2 mmol) was added at 0°C. The mixture was stirred for 3 hr at 0°C and allowed to stand overnight at room temperature. After removal of precipitate by filtration, the filtrate was evaporated and the residual oil was solidified upon addition of ether-petroleum ether. The product was recrystallized from ethyl acetate-petroleum ether; yield 690 mg (70%), mp 110°C. Other protected amino acid *p*-nitrophenylesters were synthesized similarly in 60-90% yield.

Syntheses of Acylated GS Analogs with Amino Acids —— The synthesis of [Orn(Dap)$^{2,2'}$]-GS is described as an example. To a solution of Z-Dap(Z)-ONp (100 mg, 0.2 mmol) and GS · 2HCl (66 mg, 0.05 mmol) in CH_2Cl_2 (5 ml), triethylamine (0.014 ml, 0.1 mmol) was added and stirred for 1 day at room temperature. Then, to the mixture, ethylenediamine (0.2 ml) was added, stirred for 5 hr, and evaporated. The residual oil was dissolved in ethyl acetate and the solution was washed successively with 10% citric acid, 4% $NaHCO_3$, H_2O, dried with Na_2SO_4 and evaporated. The resulting product was dissolved in a mixture of methanol (5 ml) and 1 M HCl in acetic acid (0.1 ml), and hydrogenated in the presence of Pd black for one night. After removal of the catalyst, the filtrate was evaporated, and the crude GS analog was purified by DCCC. The apparatus consisted of 150 columns. The solvent used was a mixture of ethanol-$CHCl_3$-methanol-0.1 M HCl (8 : 10 : 2 : 8, by vol.), and the lower phase of the mixture was used as a moving phase. Fractions (2.5 g) were collected and to each fraction was added methanol (1 ml) and their absorvance were determined at 250 nm. The major peak (fraction numbers 11-24) was collected and evaporated. A more detailed description of DCCC can be found in the literature (*8*). The solid was collected by filtration with the aid of ether; yield 22 mg (28%). Other analogs were

synthesized similarly in 25-30% yield. The solvent used and peak fractions for each peptide in DCCC were shown in Table I.

Synthesis of [*Orn*(*Suc*)$^{2,2'}$]-*GS* —— To a solution of GS·2HCl (50 mg, 0.044 mmol) and succinic anhydrous (22 mg, 0.22 mmol) in CH_2Cl_2 (5 ml), triethylamine (0.1 ml) was added. The mixture was stirred for one night and then added succinic anhydrous (10 mg, 0.1 mmol) and stirred for 5 hr. The mixture was evaporated and resulting product was dissolved in methanol (1 ml). The solid was collected by filtration with the aid of H_2O. The product was recrystallized from methanol-H_2O; yield 26 mg (23%).

Synthesis of [*Orn*^{2}, *Orn*(*Suc*)$^{2'}$]-*GS* —— To a solution of GS · 2HCl (270 mg, 0.18 mmol) and succinic anhydrous (26.4 mg, 0.26 mmol) in CH_2Cl_2 (10 ml), triethylamine (0.05 ml, 0.36 mmol) was added. The mixture was stirred for one night and evaporated. The resulting product was purified by DCCC; yield 17 mg (8%).

Table I. Solvents and peak fractions for each peptide

GS analogs	Solvents				Fraction numbers for each peptide
	Ethanol	$CHCl_3$	Methanol	0.1 M HCl	
[Orn(Dap)$^{2,2'}$]-GS	8	10	2	8	11 - 24
[Orn(Dab)$^{2,2'}$]-GS	10	10		7	5 - 13
[Orn(Orn)$^{2,2'}$]-GS	10	10		7	7 - 23
[Orn(Lys)$^{2,2'}$]-GS	10	10		7	16 - 38
[Orn(Ala)$^{2,2'}$]-GS		10	10	7	6 - 16
[Orn(Ser)$^{2,2'}$]-GS		10	10	7	11 - 22
[Orn(Asp)$^{2,2'}$]-GS		10	10	7	12 - 28
[Orn^2,Orn(Suc)$^{2'}$]-GS	5[a)]	10	10	4	20 - 38

a) Cyclohexane was used instead of ethanol

Antibacterial Assays —— The following microorganisms were used in the present study; *Bacillus subtilis* IFO 3007 and *Escherichia coli* B. The cells were suspended in the medium as 2.7 x 10^8 cells/ml. To each methanol solution (0.5 ml) of GS and its analogs was added the medium (9.5 ml). Various amounts of the solution were placed in test tubes and made up to 1 ml with the medium. To each tube was added the cell suspension (1 ml). After incubation for 8 hr at 30°C, water (1ml) was added and the absorvance at 620 nm was measured. More detailed description of the assay can be found in the literature (*8*).

Inhibitory Effect of GS Analogs on Adsorption of Labeled GS on Cells of B. subtilis —— Mixture (3 ml) of cold GS or analogs solution (1.5 ml) in different concentrations and cell suspension (1.5 ml) with 5.4 x10^8 cells/ml were incubated for 10 min at 30℃. Then, labeled GS solution (3 ml) in 3 μg/ml was added and the mixtures (6 ml) were incubated again for 10 min at 30°C. The mixture (5 ml) was filtered and washed with saline solution (2.5 ml x 2). The filter used was glass fiber filter, Whatman GF/B. The filter was placed in a

vial and dried *in vacuo*. Scintillater (10 ml) of 0.4% 2,5-diphenyloxazol in toluene was added to the vial, and radioactivity was measured. A control experiment was conducted without cells. The results are shown in Fig.2.

Results and Discussion

All acylated analogs of GS with amino acids were synthesized by the active ester method, and purified by DCCC. The homogeneity of obtained GS analogs was ascertained by thin-layer chromatography and elemental and amino acid analysis. All the ORD curves of the analogs were similar to that of GS and showed an intense trough at 234 nm. The result suggests that the analogs possess a preferred conformation similar to that of GS. However, among of them, the negative trough of [Orn(Asp)$^{2,2'}$]-GS [Orn(Dap)$^{2,2'}$]-GS and [Orn(Lys)$^{2,2'}$]-GS was shallower than that of GS. The result suggests that the structure of peptide-backbone of these analogs was changed even slightly.

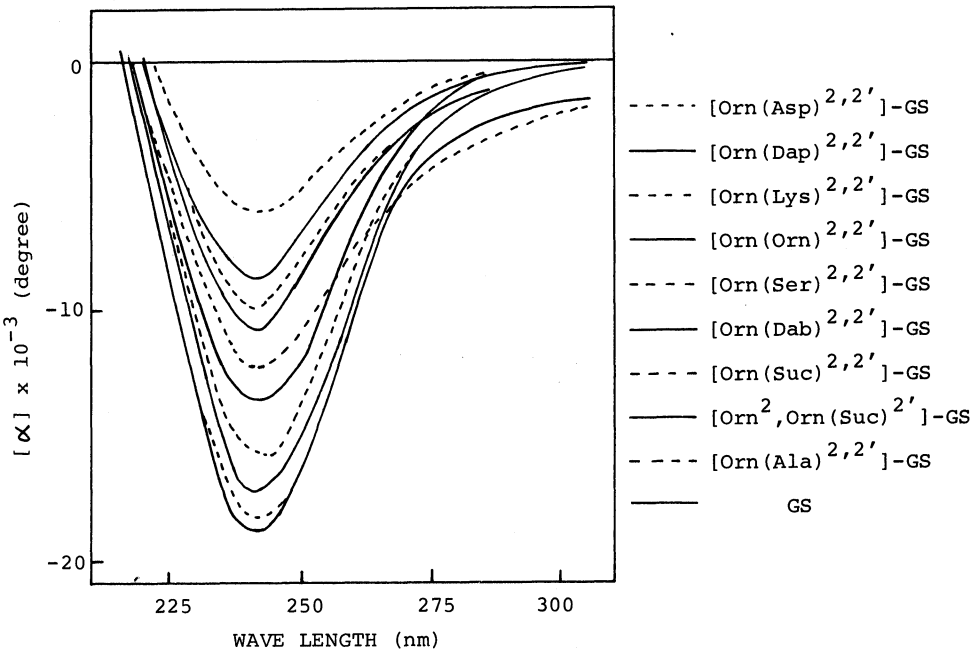

Fig. 1. ORD curves of GS analogs.

Table II shows the antibacterial activity of GS and its analogs. [Orn(Suc)$^{2,2'}$]-GS, [Orn^{2},Orn(Suc)$^{2'}$]-GS, and [Orn(Asp)$^{2,2'}$]-GS are only 1/5 - 1/10 as active to that of GS. This may be a result of decrease in positive charge.
[Orn(Dab)$^{2,2'}$]-GS, [Orn(Orn)$^{2,2'}$]-GS, [Orn(Ala)$^{2,2'}$]-GS and [Orn(Ser)$^{2,2'}$]-GS exhibited the same activity as that of GS, whereas [Orn(Dap)$^{2,2'}$]-GS and [Orn(Lys)$^{2,2'}$]-GS were 1/2 as active to that of GS.

Table II. Minimum inhibitory concentration

GS analogs	Minimum inhibitory concentrations (μg/ml)	
	B.subtilis	E.coli
GS	1.0< <1.5	5.0< <10
[Orn(Dap)$^{2,2'}$]-GS	2.0< <3.0	10< <20
[Orn(Dab)$^{2,2'}$]-GS	1.0< <1.5	5.0< <10
[Orn(Orn)$^{2,2'}$]-GS	1.0< <1.5	5.0< <10
[Orn(Lys)$^{2,2'}$]-GS	1.5< <2.0	5.0< <10
[Orn(Ala)$^{2,2'}$]-GS	1.0< <1.5	5.0< <10
[Orn(Ser)$^{2,2'}$]-GS	1.0< <2.0	5.0< <10
[Orn(Asp)$^{2,2'}$]-GS	4.0< <5.0	5.0< <10
[Orn(Suc)$^{2,2'}$]-GS	10< <20	25<
[Orn^{2},Orn(Suc)$^{2'}$]-GS	5.0< <10	25<

Then, the mode of inhibition by GS analogs of GS adsorption on cell of *B. subtilis* was studied. For this purpose, the following experiment was carried out. Cell suspension of *B. subtilis* was incubated with GS analogs in various concentrations. Then labeled GS was added in the minimum inhibitory concentration (1.5 μg/ml), the mixture incubated, and radioactivity of the cells determined. If an analog is adsorbed more strongly than GS, molecules of the analog on cells can not be replaced by GS, and therefore the amount of labaled GS on cells would be a small fraction of added labeled GS.

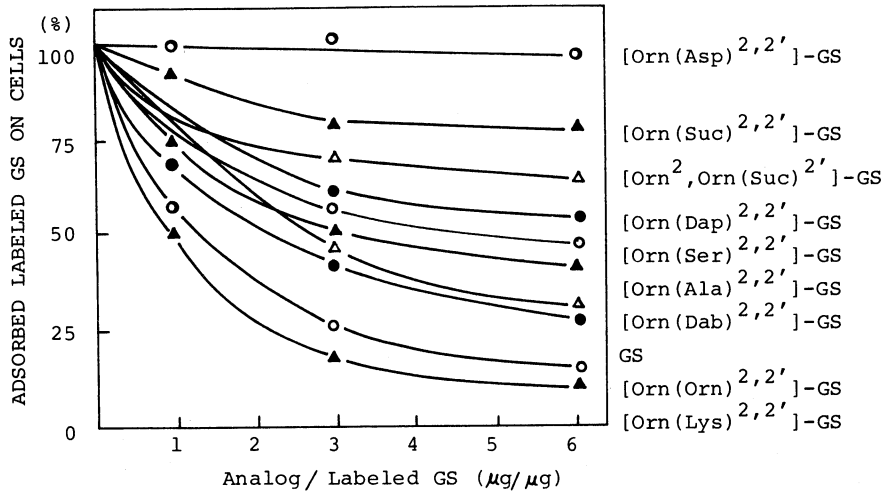

Fig. 2. Inhibitory effect of GS analogs on adsorption of labeled GS.

Fig. 2. shows the profile of inhibition by cold GS and its analogs. The results indicate that degree of inhibition parallels the antibacterial activity (Table II). It shoud be mentioned, however, that as shown in Fig. 2, the inhibitory activity on GS adsorption of [Orn(Lys)$^{2,2'}$]-GS and [Orn(Orn)$^{2,2'}$]-GS are stronger than that of cold GS, whereas as shown in Table II, the antibacterial activity of these two analogs are weaker than or equal to that of cold GS. At this time we can not explain this discrepancy. The analogs containing much positive charges than GS did not exhibit stronger antibacterial activity than GS. Consequently, two positive charges of GS are adequate for antibacterial activity of GS.

References

1. A. R. Battersby and L. C. Craig, *J. Am. Chem. Soc.*,**73**, 1887 (1951)
2. S. E. Hull, R. Karlsson, P. Main, M. M. Woolfson and E. J. Dodson, *Nature*, **275**, 206 (1978)
3. S. Hiramatsu, *J. Osaka City Med. Cent.*, (in Japanese) **9**, 4019 (1960)
4. H. Yonezawa, K. Okamoto, M. Kaneda, N. Tominaga and N. Izumiya, *Int. J. Peptide Protein Res.*, **22**, 573 (1983)
5. M. Waki, O. Abe, R. Okawa, T. Kato, S. Makisumi and N. Izumiya, *Bull. Chem. Soc. Jpn*, **40**, 2904 (1967)
6. A. B. Silaev and V. M. Stepanov, *Dokl. Akad. Nauk. SSSR*, **112**, 297 (1957)
7. V. M. Stepanov, A. B. Silaev and A. N. Polin, *Antibiotiki*, **3**, 49 (1958)
8. H. Yonezawa, M. Kaneda, N. Tominaga, S. Higashi and N. Izumiya, *J. Biochem.*, **90**, 1087 (1981)