Volume 222, number 2, 251–255 FEB 05161 October 1987

[4,4′-(Z)-Dehydrophenylalanine]gramicidin S with stabilized bioactive conformation and strong antimicrobial activity

Yasuyuki Shimohigashi, Hiroaki Kodama, Sachiko Imazu, Hideaki Horimoto*, Kazuyasu Sakaguchi, Michinori Waki, Hiroaki Uchida*, Michio Kondo*, Tetsuo Kato and Nobuo Izumiya+

*Laboratory of Biochemistry, Faculty of Science, Kyushu University 33, Fukuoka 812, *Department of Chemistry, Faculty of Science and Engineering, Saga University, Saga 840* and *+Kurume Institute of Technology, Kurume 830, Japan*

Received 14 August 1987

Dehydrophenylalanine (ΔPhe) was incorporated into an antibiotic peptide gramicidin S (GS) in place of D-Phe$^{4,4'}$ to prepare an unsaturated analog. Conformational analysis with ^{1}H-NMR indicated that the unsaturated analog has much the same backbone conformation as that of natural gramicidin S as shown by NOE experiments. Studies on temperature dependences and on the chemical shift differences showed that the hydrogen bonds between Val-NH and Leu-CO in the unsaturated analog are strengthened by the incorporation of ΔPhe$^{4,4'}$. This resulted in the reinforcement of the β-sheet structure which is the most important structural element for GS bioactivity. [ΔPhe$^{4,4'}$]gramicidin S exhibited indeed very strong antimicrobial activities against Gram-positive bacteria as well as the natural peptide.

Gramicidin S; Dehydroamino acid; Dehydropeptide; Peptide synthesis; Structure-activity relationship

1. INTRODUCTION

Simple α,β-dehydrogenation of an amino acid residue in bioactive peptides, creating an α,β-dehydroamino acid residue, has become of considerable interest due to the unique effect of this structural variation on structure-activity relationships [1]. Such modifications can elicit some conformational changes, i.e. increase in rigidity due to the limited bond angles of the residue, restriction of side chain orientations, and induction of folding. Another advantage in these modifications is the enhanced resistance of the α,β-dehydroamino acid residue to enzymatic degradations. Cyclization of the peptide sequence is also one way of restricting the conformation, and many cyclic peptides have been found to be biologically active due to a unique conformation enforced by their cyclic structure. Although α,β-dehydroamino acids have often been incorporated into bioactive peptides with a linear sequence, it is more likely that cyclization of such peptides introduces additional structural restrictions. Stabilization of bioactive conformations would provide a powerful strategy for designing an analog useful for clarifying the mechanism of bioactive ligand-receptor interactions.

Gramicidin S (GS), a cyclic decapeptide antibiotic active against Gram-positive bacteria, has a unique β-sheet structure with type II′ β-turns at the sequences of Leu-D-Phe-Pro-Val. If the D-Phe$^{4,4'}$ residues are replaced by their dehydrogenated derivative, namely dehydrophenylalanine (ΔPhe), the β-turn structure of GS

Correspondence address: Y. Shimohigashi, Laboratory of Biochemistry, Faculty of Science, Kyushu University 33, Fukuoka 812, Japan

Abbreviations: see IUPAC-IUB Commission (1984) Eur. J. Biochem. 138, 9–37. Others: ΔPhe, α,β-dehydrophenylalanine; NOE, nuclear Overhauser effect; pNA, *p*-nitroanilide; TFA, trifluoroacetic acid

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

would be rigidly constrained to stabilize the β-sheet conformation, resulting in the retention or even enhancement of GS bioactivity. Recent studies by Bach et al. [2,3] indeed indicate that the ΔPhe residue in small cyclic peptides can sustain the β- or γ-turn structure dictated by D-Phe. Thus, we have synthesized [ΔPhe$^{4,4'}$]GS (fig.1) by the conventional solution method to evaluate the effects of this replacement on both conformation and biological activity. We first synthesized Dnp-Leu-ΔPhe-Pro-Val-pNA and recorded its CD spectrum in order to assess the capability of the ΔPhe residue of forming a β-turn structure. Focusing on the structure or conformation-activity relationship, we report herein the synthesis and antimicrobial activity of [ΔPhe$^{4,4'}$]GS.

2. EXPERIMENTAL

2.1. *Peptide synthesis*

The ΔPhe moiety was introduced by spontaneous α,β-dehydrogenation and azlactonization of Boc-Leu-DL-β-phenylserine, yielding Boc-Leu-ΔPhe azlactone [4]. The *Z* configuration of the ΔPhe moiety was retained during all synthetic steps as shown by ^{1}H-NMR. The incubation of the azlactone with H-Pro-OMe yielded a tripeptide Boc-Leu-ΔPhe-Pro-OMe. For the synthesis of Dnp-Leu-ΔPhe-Pro-Val-pNA, this tripeptide was saponified to couple with H-Val-pNA. Purified Boc-Leu-ΔPhe-Pro-Val-pNA was treated with TFA, and the resulting peptide-pNA was coupled with 1-fluoro-2,4-dinitrobenzene to yield the desired Dnp-dehydrotetrapeptide-pNA. The saturated peptide Dnp-Leu-D-Phe-Pro-Val-pNA was a generous gift from Drs K. Sato and U. Nagai.

For the synthesis of [ΔPhe$^{4,4'}$]GS, the Boc-Leu-ΔPhe-Pro-OMe obtained above was elongated stepwise (50–84% yield) to afford Boc-Val-Orn(HCO)-Leu-ΔPhe-Pro-OMe by using the water-soluble carbodiimide and 1-hydroxybenzotriazole. This dehydropentapeptide ester was converted to the corresponding active ester after

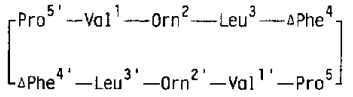

Fig.1. Primary structure of [ΔPhe$^{4,4'}$]GS.

successive saponification, esterification by *N*-hydroxysuccinimide and water-soluble carbodiimide and TFA treatment. Cyclization of the active ester H-Val-Orn(HCO)-Leu-ΔPhe-Pro-ONSu·TFA was carried out in pyridine (3×10^{-3} M). It was found that this cyclization afforded exclusively the dimeric peptide of [Orn(HCO)$^{2,2'}$,ΔPhe$^{4,4'}$]GS, as demonstrated by paper electrophoresis of the deformylated product [7]. Treatment of the Orn-formylated analog with 0.5 M HCl/MeOH gave [ΔPhe$^{4,4'}$]GS. [Orn(CHO)$^{2,2'}$]GS was prepared from natural GS by formylation with HCO-ONSu. The purity of [ΔPhe$^{4,4'}$]GS was confirmed by elemental analysis, paper electrophoresis, TLC and amino acid analysis.

2.2. *CD measurements*

CD spectra of the peptides were measured in spectrograde methanol using a Jasco J-40A automatic recording spectropolarimeter. A quartz cell with 1 mm path length was used.

2.3. *^{3}H-NMR measurements*

^{1}H-NMR spectra were recorded on a Jeol JNM-GX-270 spectrometer, operating at 270 MHz using Fourier transfer techniques. Chemical shifts were determined using tetramethylsilane as an internal standard. GS and its analogs were dissolved in dimethyl sulfoxide-d_6 (DMSO-d_6) at 10 mM for the NMR samples. The signals of protons of each peptide were assigned by two-dimensional NMR measurements using the programs whose data matrices consist of 1024 points in t_2 and 256 t_1 values zero-filled to 512 points before transformation. NOEs were measured by selectively gated irradiation (2 s) and pulse width 13.4 ms (90° flip angle).

2.4. *Microbial assays*

For determination of the minimum concentration (μg/ml) necessary for complete inhibition of various bacteria, the peptides were examined by a dilution method utilizing a trypticase soy agar.

3. RESULTS AND DISCUSSION

By measuring the CD spectra of Dnp-tetrapeptide-pNA, Sato et al. [5] have recently reported a procedure to estimate the contribution of β-turn conformation to a given tetrapeptide. It

was found that the CD spectrum of Dnp-Leu-D-Phe-Pro-Val-pNA in methanol is typical of a β-turn conformation. We then synthesized its unsaturated analog, Dnp-Leu-ΔPhe-Pro-Val-pNA, to estimate the β-turn preference of the Leu-ΔPhe-Pro-Val sequence by comparing the CD spectra of the saturated and unsaturated peptides. Although ΔPhe-containing peptide exhibited shifted Cotton effects presumably due to the ΔPhe chromophore itself, both D-Phe and ΔPhe-containing tetrapeptides showed similar CD profiles especially in the range 300–400 nm, indicating the existence of β-turn structure (fig.2). From this result it was strongly expected that fragments Leu-ΔPhe-Pro-Val in [ΔPhe$^{4,4'}$]GS, as well as its saturated fragment in natural GS, would form a β-bend to build the β-sheet backbone conformation [6].

Sequence-dependent cyclic mono- or dimerization of linear precursors having pentapeptide sequence of GS has been examined to find a sequence giving the exclusive dimeric cyclization, in relation to a position of possible β-bend in linear pentapeptide precursors [7]. In the present study, exclusive formation of the cyclic decapeptide [Orn(HCO)$^{2,2'}$,ΔPhe$^{4,4'}$]GS by cyclization of the pentapeptide H-Val-Orn(HCO)-Leu-ΔPhe-Pro-ONSu indicates further a very strong driving force of the ΔPhe-Pro sequence to form a β-bend structure.

The most important structural characteristic for antimicrobial activity of GS is the antiparallel β-sheet conformation with four intramolecular hydrogen bonds between the Val and Leu residues [6]. The β-sheet backbone conformation confers on GS the amphiphilic ring structure, in which the side chains of Val$^{1,1'}$ and Leu$^{3,3'}$ form a hydrophobic site and the side chains of Orn$^{2,2'}$ form a hydrophilic site on the other side of the ring [6]. This structural feature appears to be associated directly with the antibiotic activity. In this study, for the structural comparison of saturated and unsaturated gramicidin S, ^{1}H-NMR studies (270 MHz, in DMSO-d_6) were performed for [Orn(HCO)$^{2,2'}$]- and [Orn(HCO)$^{2,2'}$,ΔPhe$^{4,4'}$]GS and natural GS. In the NOE experiments, [Orn(HCO)$^{2,2'}$,ΔPhe$^{4,4'}$]GS showed distinct NOEs (5–20%) between protons of such pairs as ΔPhe^4 N<u>H</u> and Leu^3 C$_\alpha$<u>H</u>, Orn^2 αN<u>H</u> and Val^1 C$_\alpha$<u>H</u>, Leu^3 N<u>H</u> and Orn^2 C$_\alpha$<u>H</u>, and Val^1 N<u>H</u> and Pro^5 C$_\alpha$<u>H</u> (fig.3). These effects were also observed for saturated GS, and Rae et al. [8] and Gibbons et al. [9] have reported them as evidence in favor of the β-sheet backbone conformation of natural GS.

Temperature dependence studies further supported the conformational similarity between saturated and unsaturated gramicidin S. As shown in fig.4, all of the three peptides showed very similar temperature dependences for each amino proton. Among them, the Val$^{1,1'}$ and Leu$^{3,3'}$ amide protons exhibited small values of dependency (0.7–3.2 $\times$ 10^{-3} ppm/°C), indicating that these protons are solvent-shielded or H-bonded. It should be noted, however, that the Val$^{1,1'}$ amide protons of the unsaturated peptide have an extremely low temperature dependence (0.7 $\times$ 10^{-3} ppm/°C) as compared with those of saturated analogs (2.3–2.5 $\times$ 10^{-3} ppm/°C).

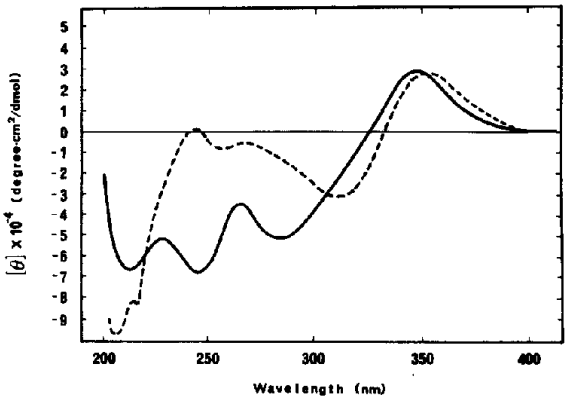

Fig.2. CD spectra of Dnp-tetrapeptide-pNA. Solvent: MeOH. Curves: (– – –) Dnp-Leu-D-Phe-Pro-Val-pNA; (——) Dnp-Leu-ΔPhe-Pro-Val-pNA.

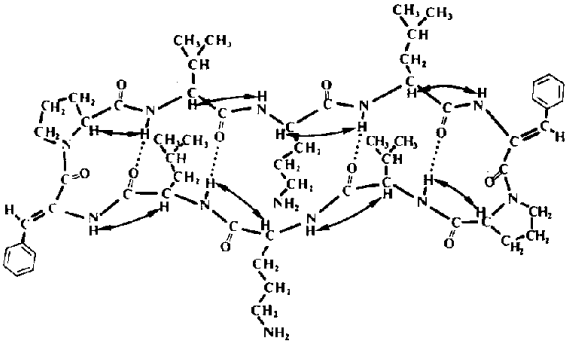

Fig.3. Conformation of [ΔPhe$^{4,4'}$]GS.

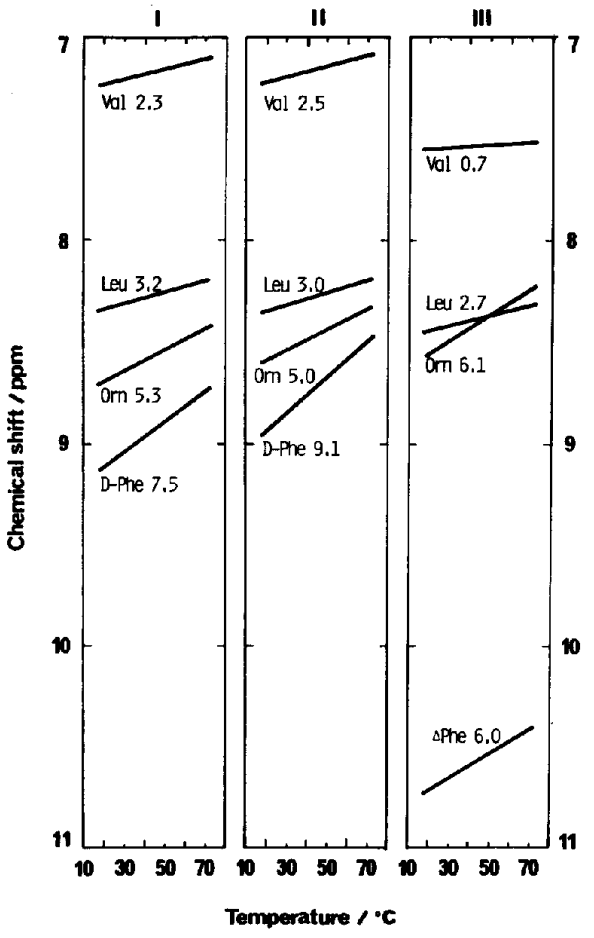

Fig.4. Temperature dependences of amide proton chemical shifts of GS·2HCl (I), [Orn(CHO)$^{2,2'}$]GS (II), and [Orn(CHO)$^{2,2'}$,ΔPhe$^{4,4'}$]GS (III) in DMSO-d_6. Numbers on the lines refer to $10^3\times$ temperature coefficient in ppm·°C^{-1}.

Moreover, from the comparative studies of the chemical shifts, it was found that the NMR spectrum of the unsaturated analog shows fairly large lower-field shifts (0.24–0.30 ppm) of Val-NH and Leu-C$_\alpha$H in addition to a very large lower-field shift (1.77 ppm) of ΔPhe-NH. These shifts should be explained by the change in electron density around the ΔPhe residue and also around the hydrogen bond between Val-NH and Leu-CO. The latter change must be due to a constrictive effect, which is caused by α,β-dehydrogenation associated with sp^3-sp^2 conversion of the ring structure of Leu-D-Phe-Pro-Val bridged by a Val-NH·Leu-CO hydrogen bond. These results together with data

Table 1

Antimicrobial activity of gramicidin S and [ΔPhe$^{4,4'}$]gramicidin S

Strain	Minimum inhibitory concentration (μg/ml)	
	GS	[ΔPhe$^{4,4'}$]GS
Staphylococcus aureus FDA 109A	6.25	6.25
Bacillus subtilis PCI 219	3.13	6.25
Shigella sonnei EW-33	25	25
Escherichia coli NIHJ JC-2	>100	>100
Escherichia coli O-111	>100	>100

on temperature dependence suggest that the hydrogen bond between Val-NH and Leu-CO is strengthened, resulting in the reinforcement of a backbone conformation of GS (fig.3). Obviously, deformylation of [Orn(HCO)$^{2,2'}$,ΔPhe$^{4,4'}$]GS yields the analog [ΔPhe$^{4,4'}$]GS having an amphiphilic nature because of the β-sheet backbone conformation.

The antimicrobial activity of GS and [ΔPhe$^{4,4'}$]GS is shown in table 1. Like natural GS, [ΔPhe$^{4,4'}$]GS is very active for Gram-positive bacteria, such as *Staphylococcus aureus* and *Bacillus subtilis*, but not for Gram-negative ones, such as *E. coli*. The potency levels shown by the minimum inhibitory concentration are almost the same for each bacterium between saturated GS and unsaturated [ΔPhe$^{4,4'}$]GS. This strong antibiotic activity of [ΔPhe$^{4,4'}$]GS must be due to a reinforced β-sheet backbone conformation with an amphiphilic nature.

The present study shows that conformational stabilization can be attained by α,β-dehydrogenation of the amino acid residue(s) of bioactive peptides. Such structural modifications clarify the molecular mechanism of interactions between peptide ligands and their receptors.

ACKNOWLEDGEMENTS

We thank Drs M. Fujino and S. Shinagawa, Takeda Chemical, Ltd, for microbial assay, and Drs K. Sato and U. Nagai, Mitsubishi Kasei Institute of Life Science, for the gift of Dnp-peptide. We also thank Dr H. Nishikawa, Fukuoka Univer-

sity, for the measurement of CD spectra, and Dr Y. Minematsu, Kao Ltd, for advice on the syntheses.

REFERENCES

[1] Shimohigashi, Y. (1986) in: Opioid Peptides: Medicinal Chemistry (Rapaka, R.S. et al. eds) NIDA Research Monograph 69, pp.65–100, NIDA-DHHS, Rockville, MD.

[2] Bach, A.C. ii and Gierash, L.M. (1985) J. Am. Chem. Soc. 107, 3349–3350.

[3] Bach, A.C. ii and Gierash, L.M. (1986) Biopolymers 25, S175–S191.

[4] Shimohigashi, Y., Stammer, C.H., Costa, T. and VonVoigtlander, P.F. (1983) Int. J. Peptide Protein Res. 22, 489–494.

[5] Sato, K., Kato, R. and Nagai, U. (1986) in: Peptide Chemistry 1985 (Kiso, Y. ed.) pp.305–310, Protein Res. Foundation, Osaka.

[6] Izumiya, N., Kato, T., Aoyagi, H., Waki, M. and Kondo, M. (1979) in: Synthetic Aspects of Biologically Active Cyclic Peptides – Gramicidin S and Tyrocidines, Kodansha, Tokyo and Wiley, New York.

[7] Minematsu, Y., Waki, M., Suwa, K., Kato, T. and Izumiya, N. (1980) Tetrahedron Lett. 21, 2179–2180.

[8] Rae, I.D., Stimson, E.R. and Scheraga, H.A. (1977) Biochem. Biophys. Res. Commun. 77, 225–229.

[9] Gibbons, W.A., Crepaux, D., Delayre, J., Dunand, J.J., Wyssbrod, H.R. (1975) in: Peptides: Chemistry, Structure and Biology (Walter, R. and Meienhofer, J. eds) pp.127–137, Ann Arbor Science, MI.