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Preparation, Stereochemistry, and Antibacterial Activity of Gramicidin S Analogs Containing *N*-Methyl Groups

Masao KAWAI,* Masashi OHYA, Norihito FUKUTA, Yasuo BUTSUGAN, and Kazuki SAITO†
Department of Appliced Chemistry, Nagoya Institute of Technology, Gokiso-cho, Showa-ku, Nagoya 466
†Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113
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The *N*-methylated analogs of gramicidin S, namely [Orn(Me_3^+)$^{2,2'}$]-, [MeOrn$^{2,2'}$, D-MePhe$^{4,4'}$]-, [MeOrn(Me)$^{2,2'}$, D-MePhe$^{4,4'}$]-, and [MeOrn(Me_3^+)$^{2,2'}$, D-MePhe$^{4,4'}$]gramicidin S, were prepared by the use of quaternization reaction of free amino groups with CH_3I–$KHCO_3$ in methanol and/or selective amide-methylation with CH_3I–Ag_2O in DMF. The conformations of these synthetic analogs were shown to be of β-sheet type similar to that of parent gramicidin S by ^1H NMR analysis and CD spectral comparison. The *N*-methyl group-containing analogs exhibited essentially the same antibacterial activity against Gram-positive bacteria as gramicidin S itself.

Gramicidin S (GS) is a cyclic decapeptide with the primary structure cyclo(–Val–Orn–Leu–D-Phe–Pro–)$_2$ showing strong antibacterial activity against Gram-positive bacteria. GS is one of the peptide antibiotics that have been most extensively studied by the synthetic approach.[1] The secondary structure of GS is established as the antiparallel β-sheet conformation having four intramolecular hydrogen bonds between two pairs of Val and Leu residues as shown in Fig. 1. Consequently the twelve nitrogen atoms present in GS are classified into four groups: two free amino groups of Orn side chains, four intramolecularly hydrogen-bonded amide NH's of Val and Leu residues, four solvent-exposed amide NH's of Orn and D-Phe residues, and two secondary amide nitrogens of Pro carrying no hydrogens. This paper deals with preparation of new analogs of GS having methyl groups on different N atoms by the application of two kinds of *N*-methylation reactions to GS or its derivatives. Stereochemistry and antibacterial activity of the *N*-methylated analogs are also described.

Preparation of Analogs

N-Methylation of a free amino group using CH_3I–$KHCO_3$ in methanol to afford a trimethylammonio group was reported by Chen and Benoiton.[2] The quaternization reaction was applied to GS to obtain a new analog of GS.[3] To the solution of GS·2HCl (**1**, 0.1 mmol) in methanol (20 ml) were added CH_3I (32 mmol) and $KHCO_3$ (10 mmol), and the mixture was stirred for 45 h. Workup and purification furnished the desired product [Orn(Me_3^+)$^{2,2'}$]GS·$2I^-$ (**2**),[4] mp 228—231.5 °C, in 75% yield, where Orn(Me_3^+) stands for a side-chain-quaternized Orn residue namely NHCH($CH_2CH_2CH_2N^+Me_3$)CO. The ^1H NMR spectrum of **2** exhibited a singlet peak at δ (DMSO-d_6) 3.11 assignable to the two trimethylammonio groups, and amino acid analysis and elemental analysis were also satisfactory.

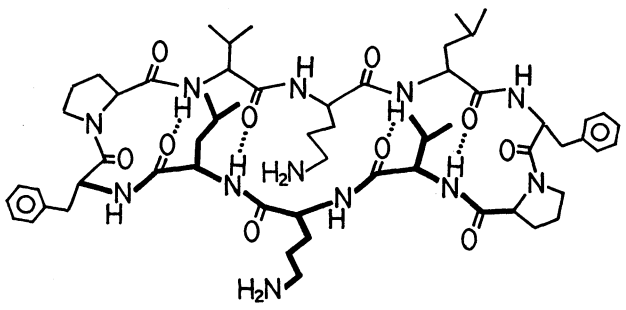
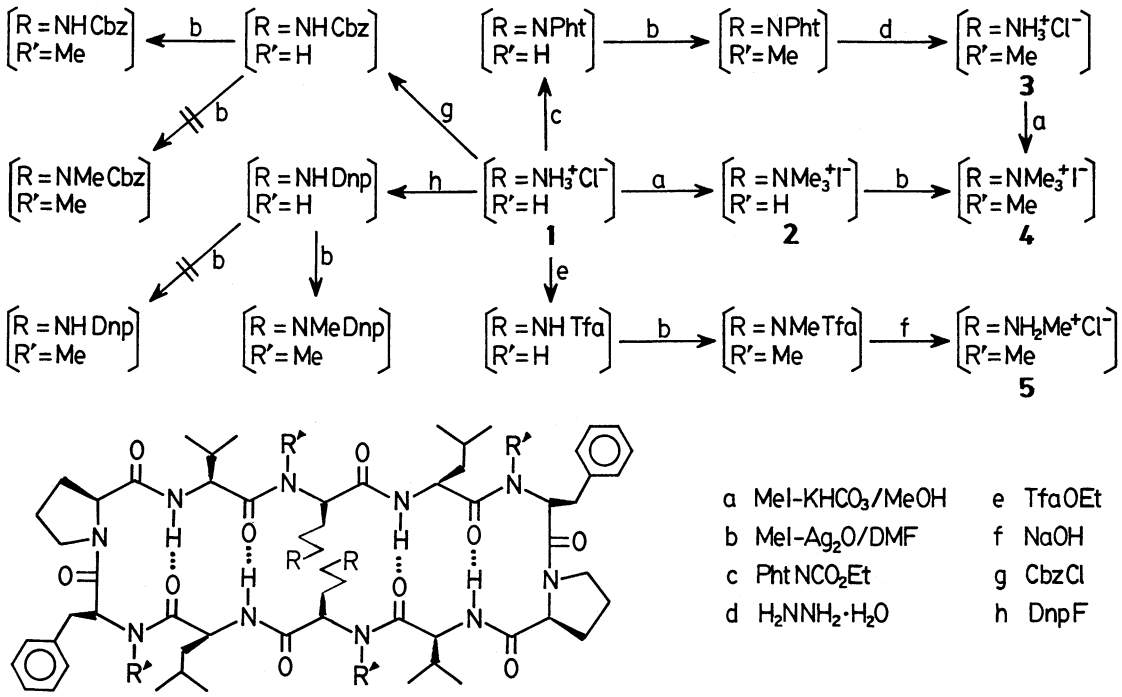


Fig. 1. β-Sheet conformation of GS with four intramolecular hydrogen bonds having C_2-symmetry.

Methylation of amide N atoms of GS was then studied. Kawai and Nagai reported conformational analysis of cyclic peptides in micromolar quantities using selective *N*-methylation with CH_3I and Ag_2O in DMF followed by amino acid analysis of the acid hydrolyzate.[5] In the case of side-chain-protected GS only solvent-exposed amide NH's of Orn and D-Phe residues were methylated while intramolecularly hydrogen-bonded amide NH's of Val and Leu residues being kept intact. The selective methylation of the diphthaloyl derivative, [Orn(Pht)$^{2,2'}$]GS, was carried out in preparative scale to yield a methylated product in 88% yield. Amino acid analysis of the acid hydrolyzate indicated that only the solvent-exposed amide NH's of Orn(Pht) and D-Phe residues had been methylated establishing the structure of the product as the desired tetramethyl derivative, namely [MeOrn-(Pht)$^{2,2'}$, D-MePhe$^{4,4'}$]GS. Removal of the phthaloyl groups with $H_2NNH_2·H_2O$ and column chromatographic purification furnished the tetramethyl analog [MeOrn$^{2,2'}$, D-MePhe$^{4,4'}$]GS·2HCl (**3**),[6] mp 200.5—203 °C, in 58% yield.

The tetramethyl analog **3** was subjected to the quaternization reaction affording a decamethyl analog [MeOrn(Me_3^+)$^{2,2'}$, D-MePhe$^{4,4'}$]GS·$2I^-$ (**4**), mp 226—232.5 °C, in 76% yield. The same compound **4** was also obtained by the application of the selective *N*-



Scheme 1.

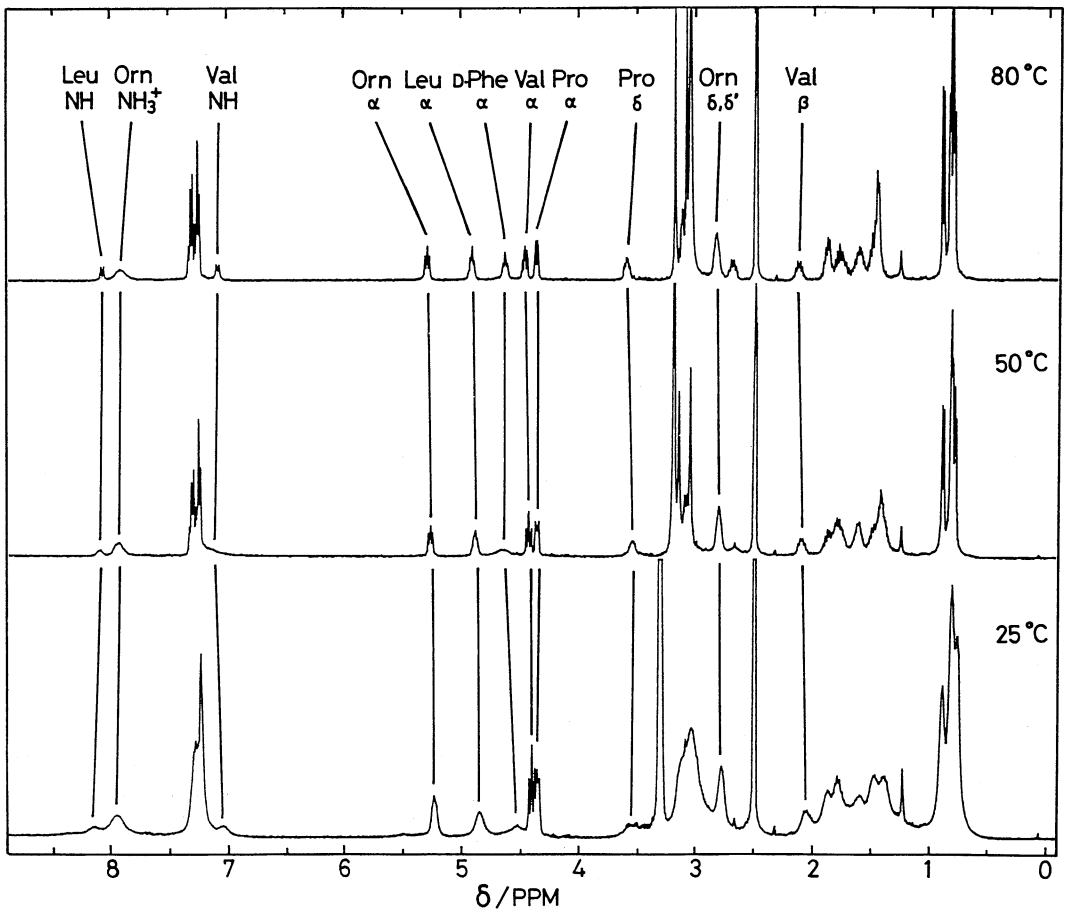


Fig. 2. 400 MHz ^{1}H NMR spectra of [MeOrn$^{2,2'}$,D-MePhe$^{4,4'}$]GS (**3**) measured in DMSO-d_6 at different temperatures.

methylation reaction to the side-chain-quaternized analog **2** in a similar manner to the *N*-methylation of the diphthaloyl derivative.

For the purpose of preparing an analog of GS having monomethylated Orn side chains, namely [MeOrn(Me)$^{2,2'}$, D-MePhe$^{4,4'}$]GS, the selective methylation reaction was applied to the bis(benzyloxycarbonyl) derivative [Orn(Cbz)$^{2,2'}$]GS. Contrary to the expectation no significant methylation at urethane NH of Orn side chain was observed, resulting in the formation of tetramethylated product [MeOrn(Cbz)$^{2,2'}$, D-MePhe$^{4,4'}$]GS. This unexpected resistance to methylation of the protected Orn side chains was noteworthy considering that the bis(2,4-dinitrophenyl) derivative [Orn(Dnp)$^{2,2'}$]GS under the same conditions afforded a hexamethyl derivative [MeOrn(Dnp,Me)$^{2,2'}$, D-MePhe$^{4,4'}$]-GS in good yield in spite of the presence of hydrogen bonding between the NH and *ortho*-NO_2 groups. The bis(trifluoroacetyl) derivative [Orn(Tfa)$^{2,2'}$]GS was found to give a hexamethyl derivative [MeOrn(Me,Tfa)$^{2,2'}$, D-MePhe$^{4,4'}$]GS on selective methylation. Alkaline hydrolysis of the trifluoroacetamido groups furnished the desired hexamethyl analog [MeOrn(Me)$^{2,2'}$, D-MePhe$^{4,4'}$]GS (**5**). Preparation of the methylated analogs **2**—**5** was summarized in Scheme 1.

Conformation of *N*-Methyl Analogs

^{1}H NMR spectral analysis of the synthetic analogs was undertaken to study their stereochemistry. The bis(trimethylammonio) analog **2** exhibited the spectra closely resembling those of GS **1**. The chemical shifts of N$^\alpha$H and coupling constants $J_{N^\alpha H\text{-}C^\alpha H}$ in the ^{1}H NMR spectrum of **2** recorded in DMSO-d_6 at 25 °C (Val δ=ca. 7.2, J=9.5 Hz; Leu δ=8.29, J=9.2 Hz; Orn δ=8.70, J=9.2 Hz; D-Phe δ=9.11, J=4.0 Hz) indicated that **2** adopted a β-sheet conformation with C_2-symmetry closely similar to that of **1**.[7] The ^{1}H NMR spectra of the tetra-*N*-methyl analog **3**, on the other hand, were apparently dissimilar to those of **1** and **2**, showing broad signals indicative of a mixture of slowly interconverting conformers. Varying-temperature study as shown in Fig. 2, however, revealed that **3** also exhibited clearly resolved sharp signals of typical β-sheet conformation at the temperatures higher than 60 °C due to rapid interconvertion of conformers. Thus, it can be assumed that although **3** exists as a comformational mixture at ambient temperature time-averaged conformation of **3** is of β-sheet type with C_2-

Table 1. 400 MHz ^{1}H NMR Spectral Data (δ/ppm) of *N*-Methylated Analogs **2**—**5** Measured in DMSO-d_6 Solutions at 80 °C

Assignments	**2**	**3**	**4**	**5**
Val				
NH[a]	7.17 (9.5)	7.08 (8.5)	7.04 (8.5)	7.18 (7.0)
C$^\alpha$H	ca. 4.40	4.46	4.41	4.47
C$^\beta$H	2.08	2.13	2.10	2.14
(C$^\gamma$H$_3$)$_2$	0.81, 0.85	0.82, 0.89	0.83, 0.92	0.83, 0.91
Orn				
N$^\alpha$H[a] or N$^\alpha$CH$_3$	8.43 (9.2)	ca. 3.17	ca. 3.1 or 3.19	3.08 or 3.18
C$^\alpha$H	4.83	5.29	5.49	5.30
C$^\beta$H$_2$	ca. 1.5, 1.76	1.88	ca. 1.83	ca. 1.76, ca. 1.92
C$^\gamma$H$_2$	ca. 1.7[b]	ca. 1.49	ca. 1.45, 1.61[b]	ca. 1.53
C$^\delta$H$_2$	3.33[b]	2.82	ca. 3.26, ca. 3.39	ca. 2.93
N$^\delta$H$_3^+$ or N$^\delta$CH$_3$	3.13	7.86	3.12	2.55
Leu				
NH[a]	8.15 (8.9)	8.07 (9.8)	8.00 (9.8)	8.09 (9.5)
C$^\alpha$H	4.59	4.91	4.92	4.92
C$^\beta$H$_2$	1.32, 1.43	ca. 1.4	ca. 1.4	ca. 1.4
C$^\gamma$H$_2$	ca. 1.5	ca. 1.4	ca. 1.4	ca. 1.4
(C$^\delta$H$_3$)$_2$	0.83	0.80, 0.83	0.81, 0.85	0.79, 0.82
D-Phe				
NH[a] or N-CH$_3$	8.76 (4.0)	ca. 3.17	ca. 3.1 or 3.19	3.08 or 3.18
C$^\alpha$H	ca. 4.40	4.63	4.56	4.70
C$^\beta$H$_2$	2.82, 2.96	ca. 3.1	ca. 3.1	ca. 3.1
arom-C$_6$H$_5$	ca. 7.25	ca. 7.3	ca. 7.3	ca. 7.3
Pro				
C$^\alpha$H	4.24	4.36	4.22	4.38
C$^\beta$H$_2$	ca. 1.61	ca. 1.77	ca. 1.78	ca. 1.76
C$^\gamma$H$_2$	ca. 1.56, 1.93[b]	ca. 1.60	ca. 1.45	1.61
C$^\delta$H$_2$	2.65, 3.59	2.68, 3.58	2.71, 3.58	2.70, 3.58

a) Coupling constant $J_{NH\text{-}CH}$ (Hz) is given in parentheses. b) Tentative assignment.

symmetry. The analogs **4** and **5** exhibited similar NMR characteristics to **3** showing well-resolved peaks at high temperatures. The ^{1}H NMR spectral data of the analogs **2**—**5** recorded at 80 °C in DMSO-d_6 solutions are summarized in Table 1, which indicates that all these analogs adopt similar conformations to that of parent **1**.

Conformation of the *N*-methyl analogs was also studied by their CD spectra measured in methanol as shown in Fig. 3. The CD spectrum of bis(trimethylammonio)-type analog **2** showing a negative maximum at 206 nm ([θ] −39500) and a shoulder at 215 nm was closely similar to that of **1**,[8] which was also consistent with their conformational similarity established by ^{1}H NMR study. The spectrum of the amide-methylated analog **3**, on the other hand, was of different pattern exhibiting a single negative maximum at the longer wavelength ([θ]$_{221}$ −37500). The difference between the spectra of **1** and **3**, however, did not indicate the conformational dissimilarity but was reasonably assumed to be due to difference of the amide chromophores, -CONH- and -CONMe-. The

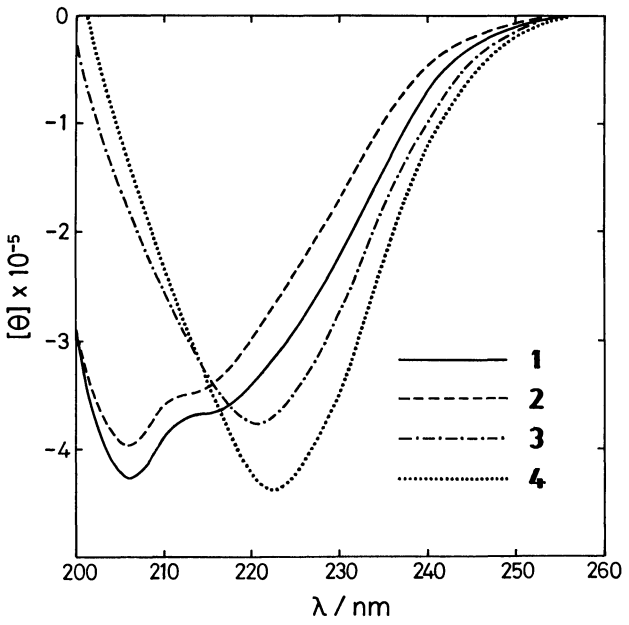


Fig. 3. CD spectra of GS·2HCl (**1**) and the *N*-methyl analogs **2**—**4** measured in MeOH at room temperature.

decamethyl analog **4** possessing also four *N*-methylated amide groups exhibited similar CD spectrum to that of **3** supporting the conformational resemblance of these analogs.

Antibacterial Activities of *N*-Methyl Analogs

Antibacterial activities of the synthetic analogs **2**—**5** were tested against several microorganisms, and minimum inhibitory concentrations of these analogs are summarized in Table 2. As expected from their conformational similarity, the *N*-methylated analogs showed essentially the same antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* strains as parent GS **1** itself.

Discussion

Synthesis of analogs containing *N*-methyl group(s) is useful for the study of the structure-activity relationships of biologically active peptides.[9] *N*-Methylamino acid-containing analogs of GS, namely [MeVal^1]GS, [MeVal$^{1,1'}$]GS, [MeLeu^3]GS, and [MeLeu$^{3,3'}$]-GS, were synthesized by Izumiya's group[10] revealing the unexpected antibacterial activities of MeLeu-containing analogs. These analogs were synthesized by the classical methods using protected *N*-methyl-amino acids as building blocks. An alternative way to prepare the *N*-methylated analogs is modification of the parent peptide molecules by the use of selective *N*-methylation reactions. In the present study two kinds of *N*-methylations, quaternization of NH_2 group into NMe_3^+ group and selective methylation of solvent-exposed -CONH- goup into -CONMe- group were employed. Preparation of enkephalin analogs by the application of the *N*-methylation reactions to suitably protected [D-Ala^2, Leu^5]enkephalin derivatives was already reported.[11]

GS derivatives possessing various groups on N^δ atoms of Orn side chains were subjected to amide-methylation and in all cases solvent-exposed N^α atoms of Orn and D-Phe were methylated while Val and Leu residues being kept intact. As an attempt to methylate the N atoms of Val and Leu residues, [Orn(Pht)$^{2,2'}$]GS was subjected to prolonged methylation with larger amount of reagents still resulting in the isolation of only the tetramethyl derivative [MeOrn(Pht)$^{2,2'}$, D-MePhe$^{4,4'}$]GS. These results indicated that the

Table 2. Antibacterial Activities of GS **1** and Its *N*-Methyl Analogs **2**—**5** (Minimum Inhibitory Concentrations in μg cm^{-3})

Microorganism	1	2	3	4	5
Staphylococcus aureus FDA 209 PJC-1	1.56	1.56	1.56	1.56	1.56
Staphylococcus aureus Terajima	3.1	1.56	3.1	3.1	3.1
Bacillus subtilis ATCC 6633	1.56	1.56	1.56	3.1	1.56
Klebsielle pneumoniae PCI-602	12.5	12.5	50.0	>100	50.0
Escherichia coli K 12 C 600	50.0	50.0	50.0	>100	100
Escherichia coli NIHJ-JC-2	50.0	50.0	100	>100	100

intramolecularly hydrogen-bonded β-sheet conformation of GS is very stable regardless of the various modification of Orn side chains and also that the stability of the conformation is maintained during and after the methylation of solvent-exposed amide-N atoms.

It is quite interesting that the side chains of Orn protected with benzyloxycarbonyl groups in GS were intact during the amide-methylation being in contrast to the methylation of [Orn(Tfa)$^{2,2'}$]GS and [Orn(Dnp)$^{2,2'}$]GS, in which N^{δ} atoms were also methylated in good yield. Model reaction using Ac–Orn(Cbz)–OMe and Ac–Lys(Cbz)–OMe under the same conditions revealed that both urethane NH in the side chain and acetamido groups were methylated without difficulty yielding Ac–MeOrn(Cbz,Me)–OMe and Ac–MeLys(Cbz,Me)–OMe, respectively. The results suggested the presence of some interaction between Orn side chains and the rest of the molecule in [Orn(Cbz)$^{2,2'}$]-GS. Hydrogen bonding of one of the Orn side chains with the CO group of Phe residue was actually observed for GS-urea complex in crystalline state.[12]

NMR and CD spectral studies on conformational characteristics of the synthetic analogs showed the conformational resemblance of the *N*-methyl analogs **2—5** and parent GS **1**. ^{1}H NMR spectra of the amide-methylated analogs **3—5** measured in DMSO-d_6 solution at 25 °C exhibited broad signals due to slow conformational interconversion. Since sharp signals assignable to β-sheet conformation were observed at higher temperature as a result of rapid interconvertion among conformers, averaged conformation at the low temperature was also considered to resemble the conformation of **1**. In fact ^{1}H NMR spectrum of **3** measured in $CDCl_3$ at 25 °C exhibited well-resolved sharp signals characteristic of GS-type β-sheet conformation. Thus, it can be concluded that all these GS analogs possess essentially the same conformational characteristics as GS and that methyl groups at the solvent-exposed N atoms cause slight decrease in conformational mobility resulting in the slow conformational interchange in strongly solvating DMSO solution. This also suggests that the well-established β-sheet conformation having C_2-symmetry as shown in Fig. 1 of GS and its derivatives can be considered as an averaged structure of the assembly of slightly different conformers in equilibrium.[13]

From the intensive studies on the structure-antibacterial activity relationships of the analogs of GS it is known that both the stable β-sheet conformation of GS and the presence of positively charged side chains are important for the activity. As given in Table 2 the *N*-methylated analogs **2—5** showed very similar activity to that of parent **1**, which is consistent with the conformational similarity of all these analogs as described above. As for the Orn side chains the essentially same activity of the trimethylammonio analog **2** as **1** has demonstrared that when these antibiotics bind to bacterial membrane or receptor molecules the function of the charged Orn side chains does not involve interaction through hydrogen bonding but is simply electrostatic in nature. The activity of **3—5** has also indicated that the exposed amide NH's of Orn and D-Phe residues do not play any important role in the binding of GS molecules to bacteria and in manifesting the antibactrial activity.

Experimental

GS·2HCl (**1**) was supplied by Nikken Kagaku Ltd. Mp's were determined with a hot-stage apparatus and uncorrected. Silica gel (Merck, #7734) and Sephadex LH-20 were used for column chromatographic separation. Purity of each product was ascertained by TLC, HPLC, and/or ^{1}H NMR spectral analysis. TLC analysis was performed using precoated silica gel plates (Merck, Silica Gel 60 F_{254}) with the solvent systems $CHCl_3$–MeOH, $CHCl_3$–MeOH–AcOH, or 1-butanol–AcOH–H_2O. HPLC was performed using FineSIL 20 (JASCO) with $CHCl_3$–MeOH as eluent or Unisil C18 (Gasukuro Kogyo) with MeOH–$NaClO_4$ aq. ^{1}H NMR spectra were recorded on a JEOL GX-400 or on a Varian XL-200 spectrometer. CD spectra were measured on a JASCO J-40C spectropolarimeter. For amino acid analysis synthetic peptides were hydrolyzed in concd HCl–AcOH (1:1) at 110 °C for 24 h. Proline was taken as an internal standard, and Orn(Me_3^+) and MeOrn were determined by the comparison with the authentic mixture. Antibacterial assay was performed at Research Center, Mitsubishi Kasei Corporation, and elemental analysis was performed at Elemental Analysis Center of Kyoto University.

[Orn(Me_3^+)$^{2,2'}$]GS·2I$^-$ (2). CH_3I (2 cm^3, 32 mmol) and $KHCO_3$ (1.0 g, 10 mmol) were added to the solution of **1** (125 mg, 0.1 mmol) in MeOH (20 cm^3) and the mixture was stirred in the dark for 45 h at room temperature. The solvent was evaporated and the residue was extracted with $CHCl_3$. Sephadex LH-20 column chromatography of the $CHCl_3$ extract using MeOH as eluent and recrystallization from MeOH–H_2O afforded **2** (116 mg, 75%) as colorless needles, mp 228—231.5 °C. Amino acid analysis: Pro (1.00), Val (1.02), Leu (0.97), Phe (1.00), Orn (0.00), Orn (Me_3^+) (1.00).

Found: C, 50.67; H, 7.52; N, 10.89%. Calcd for $C_{66}H_{106}N_{12}O_{10}I_2 \cdot 5H_2O$: C, 50.44; H, 7.44; N, 10.70%.

[MeOrn(Pht)$^{2,2'}$, D-MePhe$^{4,4'}$]GS. [Orn(Pht)$^{2,2'}$]GS (42 mg, 0.03 mmol), prepared from **1** and *N*-(ethoxycarbonyl)phthalimide,[14] was dissolved in DMF (1 cm^3), and CH_3I (2.5 cm^3, 40 mmol) and Ag_2O (232 mg, 1.0 mmol) were added to the solution. After stirring for 4 h at room temperature MeOH (4 cm^3) was added and the precipitate was filtered off. The solvent was evaporated and the residue was extracted with $CHCl_3$. Silica-gel column chromatography of the $CHCl_3$ extract using $CHCl_3$–MeOH as eluent and crystallization from MeOH–H_2O afforded [MeOrn(Pht)$^{2,2'}$, D-MePhe$^{4,4'}$]GS (38.5 mg, 88%), mp 170.5—173.5 °C. ^{1}H NMR ($CDCl_3$) δ=3.21 (6H, s, N–Me) and 3.52 (6H, s, N–Me). Amino acid analysis: Pro (1.00), Val (1.00), Leu (0.95), Phe (0.00), Orn (0.00), MeOrn (1.05).

Found: C, 64.93; H, 7.09; N, 11.56%. Calcd for $C_{80}H_{104}N_{12}O_{14}I_2 \cdot H_2O$: C, 65.11; H, 7.24; N, 11.39%.

[MeOrn$^{2,2'}$, D-MePhe$^{4,4'}$]GS·2HCl (3). $H_2NNH_2 \cdot H_2O$ (5

cm³) was added dropwise to the solution of [MeOrn(Pht)$^{2,2'}$, D-MePhe$^{4,4'}$]GS (73 mg, 0.05 mmol) in EtOH (2 cm³) and the mixture was stirred overnight at room temperature. After the addition of H_2O (10 cm³) precipitate was collected by filtration and was dissolved in EtOH (5 cm³), to which 1 M (M=mol dm^{-3}) HCl aq (1 cm³) was added. The solvent was evaporated and the residue was chromatographed with silica gel ($CHCl_3$–MeOH–AcOH) and then with Sephadex LH-20 (MeOH) affording **3** (37 mg, 58%), mp 200.5—203 °C.

Found: C, 55.97; H, 8.37; N, 11.53%. Calcd for $C_{64}H_{100}N_{12}O_{10}\cdot 2HCl\cdot 3CH_3OH\cdot 4H_2O$: C, 55.94; H, 8.55; N, 11.68%.

[MeOrn(Me$_3^+$)$^{2,2'}$, D-MePhe$^{4,4'}$]GS·2I$^-$ (4) Quaternization of **3** (63 mg 0.05 mmol) was undertaken in a similar manner to the preparation of **2** from **1**. Sephadex LH-20 column chromatography (MeOH) of the product and crystallization from MeOH–Et_2O–hexane gave **2** as colorless needles (57 mg, 76%), mp 226—232.5 °C. Amino acid analysis: Pro (1.00), Val (0.99), Leu (1.06), Phe (0.00), Orn (0.00).

Found: C, 52.13; H, 7.24; N, 10.47%. Calcd for $C_{70}H_{114}N_{12}O_{10}I_2\cdot 4H_2O$: C, 52.23; H, 7.64; N, 10.44%.

N-Methylation of **2** in a similar manner to the preparation of [MeOrn(Pht)$^{2,2'}$, D-MePhe$^{4,4'}$]GS from [Orn(Pht)$^{2,2'}$]GS afforded the same compound **4** in 32% yield.

[Orn(Tfa)$^{2,2'}$]GS. Ethyl trifluoroacetate (3 cm³) was added to the solution of **1** (121 mg, 0.1 mmol) and Et_3N (0.03 cm³, 0.2 mmol) in MeOH (5 cm³), and the mixture was stirred overnight at room temperature. The solvent was evaporated and the residue was dissolved in $CHCl_3$ and washed with H_2O. Evaporation of $CHCl_3$ and crystallization of the residue from MeOH–H_2O gave [Orn(Tfa)$^{2,2'}$]GS as colorless prisms (112 mg, 83%), mp 273 °C (decomp).

Found: C, 56.49; H, 6.89; N, 12.40%. Calcd for $C_{64}H_{90}N_{12}F_6O_{12}\cdot 1.5H_2O$: C, 56.50; H, 6.89; N, 12.39%.

[MeOrn(Me,Tfa)$^{2,2'}$, D-MePhe$^{4,4'}$]GS. *N*-Methylation of [Orn(Tfa)$^{2,2'}$]GS (45 mg, 0.033 mmol) was carried out in a similar manner to the preparation of [MeOrn(Pht)$^{2,2'}$, D-MePhe$^{4,4'}$]GS from [Orn(Pht)$^{4,4'}$]GS. Silica-gel column chromatography ($CHCl_3$–MeOH) of the product and crystallization from MeOH–H_2O gave [MeOrn(Me,Tfa)$^{2,2'}$, D-MePhe$^{4,4'}$]GS (39 mg, 83%), mp 125—126.5 °C. ^{1}H NMR ($CDCl_3$) δ=3.14 (6H, s, *N*-Me), 3.25 (6H, s, *N*-Me), and 3.41 (6H, s, *N*-Me). Amino acid analysis: Pro (1.00), Val (0.98), Leu (0.96), Phe (0.00), Orn (0.00).

[MeOrn(Me)$^{2,2'}$, D-MePhe$^{4,4'}$]GS·2HCl (5). To the solution of [MeOrn(Me,Tfa)$^{2,2'}$, D-MePhe$^{4,4'}$]GS (53 mg, 0.043 mmol) in MeOH (3 cm³) was added 4M NaOH aq (0.7 cm³) and the mixture was stirred for 2 h at room temperature. The solution was acidified by the addition of 6M HCl aq and was evaporated, and the residue was extracted with $CHCl_3$. Sephadex LH-20 column chromatography (MeOH) of the $CHCl_3$ extract afforded **5** (40 mg, 82%), mp 175—177.5 °C.

Found: C, 56.72; H, 8.21; N, 12.09%. Calcd for $C_{66}H_{104}N_{12}O_{10}\cdot 2HCl\cdot 5.5H_2O$: C, 56.72; H, 8.44; N, 12.03%.

***N*-Methylation of [Orn(Cbz)$^{2,2'}$]GS.** [Orn(Cbz)$^{2,2'}$]GS[15] (127 mg, 0.09 mmol) was subjected to the *N*-methylation reaction in a similar manner to the preparation of [MeOrn(Pht)$^{2,2'}$, D-MePhe$^{4,4'}$]GS from [Orn(Pht)$^{4,4'}$]GS. The crude product showed a prominent single spot on TLC accompanied with some minor spots, and silica-gel column chromatography ($CHCl_3$–MeOH) and crystallization from MeOH–H_2O yielded [MeOrn(Cbz)$^{2,2'}$, D-MePhe$^{4,4'}$]GS (103 mg, 76%), mp 127.5—129.5 °C. Amino acid analysis: Pro (1.00), Val (1.01), Leu (0.93), Phe (0.00), Orn (0.00), MeOrn (1.01).

***N*-Methylation of [Orn(Dnp)$^{2,2'}$]GS.** [Orn(Dnp)$^{2,2'}$]GS[16] (74 mg, 0.05 mmol) was subjected to the *N*-methylation reaction as described above. Silica-gel column chromatography ($CHCl_3$–MeOH) afforded [MeOrn(Me,Dnp)$^{2,2'}$, D-MePhe$^{4,4'}$]GS (73 mg, 95%), mp 135—138 °C. ^{1}H NMR ($CDCl_3$) δ=2.88 (s, 6H, *N*-Me), 3.19 (s, 6H, *N*-Me), and 3.38 (s, 6H, *N*-Me).

Found: C, 58.72; H, 6.85; N, 13.77%. Calcd for $C_{78}H_{108}N_{16}O_{18}\cdot 2H_2O$: C, 58.78; H, 7.08; N, 14.06%.

2,4-Dinitrophenylation of **4** (15 mg, 0.012 mmol) in EtOH (1 cm³) with DnpF (105 mg, 0.56 mmol) and Et_3N (0.05 cm³) for 2 h at room temperature also yielded the same compound, mp 135—136 °C, in 87% yield.

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