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Stereochemistry of Protected Ornithine Side Chains of Gramicidin S Derivatives: X-ray Crystal Structure of the Bis-Boc-tetra-*N*-methyl Derivative of Gramicidin S

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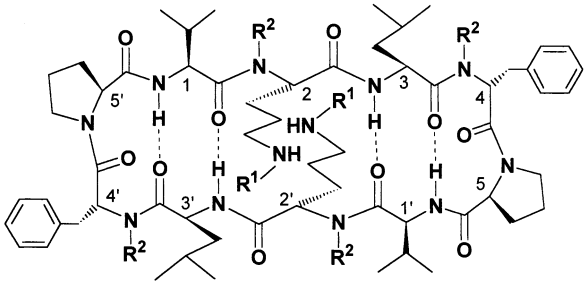
Abstract: An X-ray crystallographic analysis of the bis-N^{δ}-Boc-tetra-N^{α}-methyl derivative of gramicidin S, *cyclo*(−Val−MeOrn(Boc)−Leu−D-MePhe−Pro−)$_2$, was undertaken successfully (*R*-factor = 0.088). As expected, the main chain adopts an antiparallel pleated β-sheet conformation, but the pleated sheet is slightly twisted, and the sense of twisting is opposite to that found in the reported crystal structures of the gramicidin S-urea complex and the bis-N^{δ}-(trichloroacetyl) and bis-N^{δ}-(*m*-bromobenzoyl) derivatives of gramicidin S. In agreement with the observed resistance toward N-methylation, the urethane NH groups of the protected Orn side chains are hydrogen bonded to the carbonyl groups of the D-Phe residues. However, the side-chain−main-chain hydrogen bonding is in the $i \rightarrow i-3$ mode, although hydrogen bonding in the $i \rightarrow i+2$ mode was deduced from a ^{1}H NMR study of protected gramicidin S derivatives and was actually found in the crystal structures of the diacylated gramicidin S.

Introduction

A wide variety of methods and techniques are available for studying the stereostructure of peptides, as well as other organic compounds.[1] Among them, X-ray crystallography is unique because it affords unambiguous definitive information concerning conformations in the crystal state which could contribute to the understanding of stereochemical properties in solution. Gramicidin S (GS) is a cyclic decapeptide antibiotic with two identical pentapeptide sequences (Figure 1), *cyclo*(−Val−Orn−Leu−D-Phe−Pro−)$_2$.[2] Even after the main-chain conformation of GS was shown to be the antiparallel β-sheet with the D-Phe−Pro sequences at the type II′ β-turn moieties,[3] this molecule has still been subjected to numerous stereochemical analyses using various methodologies.[4−6] The main-chain conformation of GS is so distinct and stable that GS and its derivatives are quite useful as model compounds for testing new NMR methods for conformational analysis.[4] Although stereochemical studies of GS and related compounds are abundant, crystallographic studies are few in number,[7] and the X-ray single-crystal structural analysis of the hydrated GS-urea complex[7d,e] in 1978 was the only successful example until the crystal structures of the two diacylated derivatives, bis-N^{δ}-(trichloroacetyl) GS and bis-N^{δ}-(*m*-bromobenzoyl) GS, were reported recently.[7f]



$R^1 = R^2 = H$ gramicidin S (GS) = *cyclo*(-Val-Orn-Leu-D-Phe-Pro-)$_2$

R^1 = Boc, R^2 = CH_3 [MeOrn(Boc)$^{2,2'}$,D-MePhe$^{4,4'}$]GS (**1**)

Figure 1. Structure of GS and N-methylated derivative **1**.

It has been been shown that peptide conformations can be studied using the chemical reactivity of amide functions.[6]

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Table 1. Crystallographic Data for **1**·DMSO-d_6·4H_2O

formula (fw)	$C_{76}H_{116}N_{12}O_{14}$·$C_2D_6SO$·4$H_2O$ (1554.02)
crystal color, habit	colorless, prism
crystal dimensions	0.40 × 0.40 × 0.20 mm
crystal system, space group	orthorhombic, $P2_12_12_1$ (No. 19)
no. of reflections (2θ range)	34 062 (2.0−65.0°)
lattice parameters	a = 11.201(1) Å, b = 19.401(2) Å, c = 40.438(6) Å, V = 8787(1) Å^3
Z value	4
D_{calc}	1.170 g/cm^3
F_{000}	3352.00
μ (Mo Kα)	1.06 cm^{-1}
diffractometer	Rigaku RAXIS-IV imaging plate
radiation	Mo Kα (λ = 0.70920 Å) graphite monochromated
temperature	−20 ± 1 °C
oscillation range, $2\theta_{max}$	5.0°, 64.6°
no. of reflections measured	total: 30 373, unique: 12 956 (R_{int} = 0.046)
structure solution	direct methods (SIR92)
refinement	full-matrix least-squares (SHELXL-97)
function minimized	$\sum w(F_o^2 - F_c^2)^2$
least squares weights	$w = 1/[\sigma^2(F_o^2) + (0.1000P)^2 + 0.0000P]$ where $P = (F_o^2 + 2F_c^2)/3$
no. of reflections (all, $2\sigma < 55.00°$)	16 096
no. variables	947
reflection/parameter ratio	17.00
residuals: R; R_w	0.088; 0.244
GOF indicator	1.46
max. shift/error in final cycle	−0.05
peak height in final diff. map	0.75, −0.66 e/Å^3

N-Methylation of amide functions with CH_3I-Ag_2O in DMF was used for the determination of the hydrogen-bonded amide functions of cyclic peptides including GS[6a] and was applied to the preparation of new GS analogues.[8] While the solvent-exposed α-NHs of the Orn and D-Phe residues in GS were easily methylated in this system, four intramolecularly hydrogen-bonded α-NHs on the Val and Leu residues were unreactive. The urethane NHs of protected Orn side chains were also found to be resistant to N-methylation, due to hydrogen bonding with the carbonyl groups of the D-Phe residues[9] to afford tetra-N-methylated products.[8b] However, the N-methylated bis-Boc derivative of GS was found to be suitable for single-crystal X-ray crystallographic analysis. Here we describe the crystal structure of this unique multiply N-substituted derivative, which revealed an unexpected mode of hydrogen bonding between the substituted Orn side chains and the main chain.

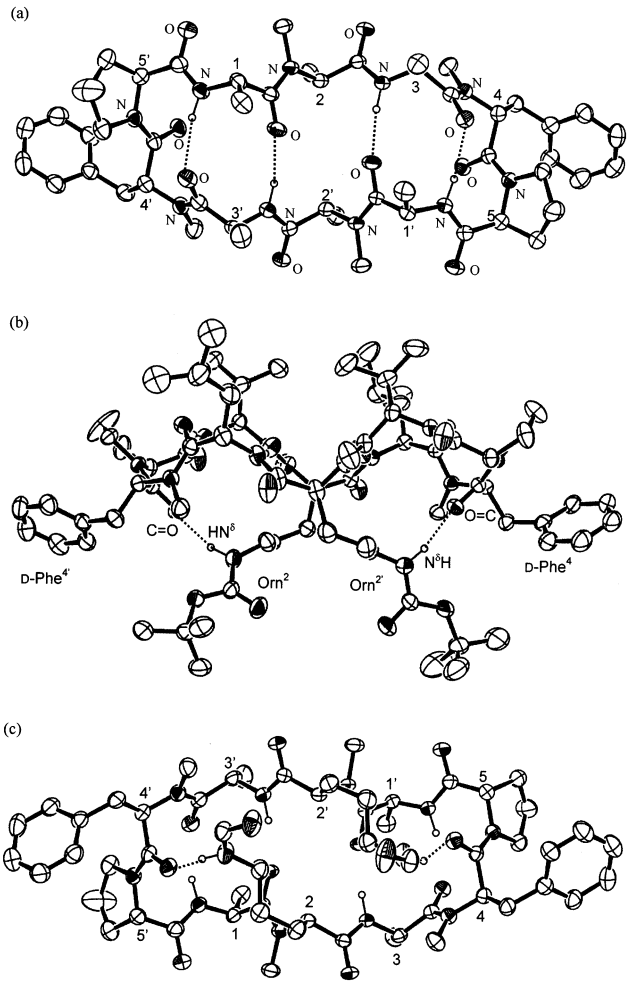

Figure 2. ORTEP diagram of **1** showing 30% thermal ellipsoids. (a) Top view, (b) lateral view, and (c) bottom view. For clarity, side-chain atoms of Val, Orn, and Leu except β-carbons are omitted in (a), and side chains of Val and Leu except β-carbons and *tert*-butoxy groups in Orn(Boc) are omitted in (c). Antiparallel β-sheet-type hydrogen bonds are shown in (a), and side-chain−main-chain hydrogen bonds in (b) and (c) are shown by broken lines. Only the hydrogen atoms participating in intramolecular hydrogen bonding are shown.

Results

The tetra-N-methylated bis-Boc derivative of GS (Figure 1), [MeOrn(Boc)$^{2,2'}$,D-MePhe$^{4,4'}$]GS (**1**), was synthesized as an intermediate for the preparation of the unsymmetrical [MeOrn-(Boc)^2,MeOrn(PyCO)$^{2'}$,D-MePhe$^{4,4'}$]GS (PyCO = 2-pyridinecarbonyl).[10] The derivative **1** was subjected to NMR measurements, and, after standing for years, well-defined colorless crystals crystallized out from the DMSO-d_6 solution of **1**.

The crystallographic data are summarized in Table 1, and the molecular structure of **1** in the crystal is shown in Figure 2. The molecule is approximately C_2 symmetrical, except for the side chains of the Leu residues, as demonstrated by the torsion angle values given in Table 2. The molecule adopts a slightly distorted β-sheet conformation, in which the hydrogen-bonded N···O distances are 3.03 and 3.04 Å for NH(Val)···C═O(Leu)

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Table 2. Torsion Angles of **1** and Other GS Derivatives in Crystals and Those Obtained by Theoretical Calculations

				in crystal						calculated[e]	
residue	angle	**1**[a]		GS-urea complex[b]		[Orn(Tca)$^{2,2'}$]GS[c,d]		[Orn(*m*-BrBz)$^{2,2'}$]GS[c,d]		GS[f]	GS[g]
Val	ϕ	−102.8(3)	−101.2(3)	−120	−125	−109	−114	−122	−99	−90	−90
	ψ	108.6(3)	105.4(3)	157	153	131	116	129	125	100	131
	ω	−179.5(3)	176.3(3)	172	174	171	176	178	174	(180)	(180)
	χ^{11}	−61.0(4)	−59.8(4)	−63	59	−67	−67	−59	−55	178	−178
	χ^{12}	178.7(3)	177.6(3)	−177	−71						
Orn	ϕ	−124.5(3)	−122.5(3)	−108	−105	−117	−112	−122	−131	−127	−147
	ψ	97.4(3)	90.9(3)	131	136	131	128	128	119	125	126
	ω	−173.9(2)	−169.0(3)	177	178	174	175	179	178	(180)	(180)
	χ^{1}	−55.1(4)	−55.2(4)	−174	−66	−163	180	−174	−178	−63	166
	χ^{2}	−64.9(4)	−66.2(5)	179	173	175	65	−153	174	−170	72
	χ^{3}	−173.5(3)	−176.5(3)	−166	167	−171	170	−180	−174	−177	166
	χ^{4}	115.6(4)	91.2(5)			135	−110	151	154	25	60
Leu	ϕ	−127.4(3)	−125.3(3)	−121	−139	−125	−124	−130	−114	−156	−154
	ψ	81.0(3)	84.3(3)	93	121	93	97	101	109	117	114
	ω	170.0(2)	173.0(3)	−178	−173	−177	−178	179	−179	(180)	(180)
	χ^{1}	−72.9(4)	−156.0(3)	−60	−72	−76	−59	−59	−57	−179	−175
	χ^{21}	−78.6(5)	−57.6(4)	−143	−65	−82	−58	−180	−54	72	75
	χ^{22}	162.9(5)	162.6(3)	−62	177	156	179	−53	−178		
D-Phe	ϕ	67.6(3)	64.6(3)	61	58	58	63	62	54	60	59
	ψ	−125.1(2)	−127.9(3)	−126	−136	−128	−130	−133	−124	−137	−137
	ω	−177.9(2)	−177.6(3)	−174	−177	−174	−174	−171	−172	(180)	(180)
	χ^{1}	176.1(2)	179.1(3)	174	176	177	−177	179	177	179	172
	χ^{2}	−83.2(4)	−87.3(4)	−75	−85	−97	−91	−88	−84	−91	−89
Pro	ϕ	−75.0(3)	−72.0(4)	−81	−93	−83	−82	−79	−94	(−75)	(−75)
	ψ	−0.9(4)	−7.3(4)	−2	11	9	−2	−10	15	−18	8
	ω	175.8(3)	−177.3(3)	−174	−173	178	−178	−173	178	(180)	(180)
	χ^{1}	27.2(4)	16.1(6)	21	38	−6	34	−10	38	(19)	(19)
	χ^{2}	−31.0(5)	−15.5(9)	−20	−37	−40	−40	−39	−42	(−14)	(−14)
	χ^{3}	21.4(4)	8.0(9)		22	28	30	29	28	(4)	(4)

[a] Standard deviations are given in parentheses. Left, residues 1−5; right, residues 1′−5′. [b] Reference 7e. [c] Reference 7f. [d] Tca = trichloroacetyl, *m*-BrBz = *m*-bromobenzoyl. [e] C_2 symmetrical structure was assumed. The values in parentheses were not optimized but treated as fixed. [f] Reference 5a. [g] Reference 5d.

and 2.79 and 2.86 Å for NH(Leu)···C═O(Val). The main-chain antiparallel pleated β-sheet is slightly twisted; that is, the D-Phe and Pro residues are dislocated toward the hydrophilic and hydrophobic sides (downward and upward), respectively.

The most interesting feature of the crystal structure of **1** is the orientation of the Orn side chains. As deduced from the N-methylation study, a side-chain−main-chain hydrogen-bonding interaction is observed between the Orn and D-Phe residues, the N···O distance for N$^{\delta}$H(Orn) and C═O(D-Phe) being 2.83 and 2.87 Å. However, the hydrogen bonds of **1** are of the $i \rightarrow i - 3$ mode, that is, between the Orn^{2} and D-Phe$^{4'}$ and between the Orn$^{2'}$ and D-Phe^{4} residues, which are opposite to those determined from the ^{1}H NMR study using unsymmetrical GS derivatives,[9] that is, in the $i \rightarrow i + 2$ mode between the Orn^{2} and D-Phe^{4} and between the Orn$^{2'}$ and D-Phe$^{4'}$ residues.

The molecular packing of **1** in the crystal is shown in Figure 3. Reflecting the thin, sheetlike structure of the molecule **1**, the lattice has a relatively short *a*-axis and long *c*-axis. Four molecules of **1**, four DMSO molecules, and 16 water molecules are included in a single lattice. The packing diagram, with possible intermolecular hydrogen bonding, is shown in Figure 4. Because of the presence of the N^{α}-methyl groups on the Orn and D-Phe residues, no cross-β-sheet hydrogen bonds were found, as observed in the diacyl GS.[7f] Two molecules of **1** are connected by hydrogen bonding via three molecules of water. Among the three water molecules, one is hydrogen bonded to two carbonyl groups of the Boc groups on the Orn side chains, while another molecule is hydrogen bonded to the carbonyl group of the second molecule of **1**, constituting the hydrogen-bonding network. The fourth water molecule is located at hydrogen-bonding distance from the carbonyl group of the Pro′ residue. These water molecules, as well as DMSO molecules, are also used for filling the space in the lattice.

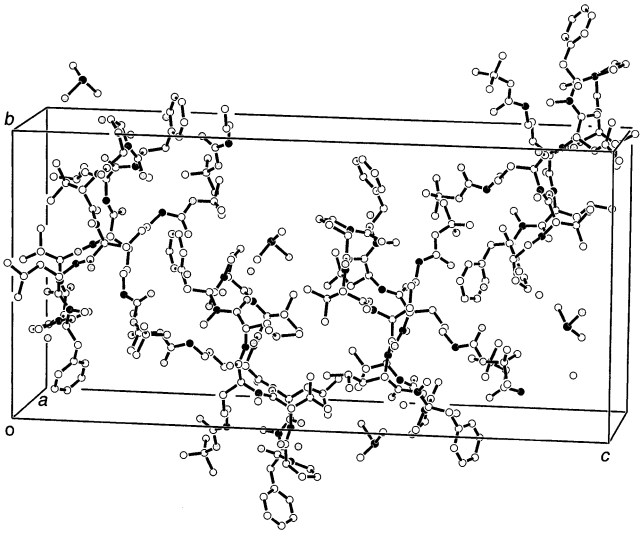

Figure 3. Molecular packing of **1** in the crystal. Four molecules of **1**, four molecules of DMSO, and 16 water molecules are shown. Hydrogen atoms are omitted.

Discussion

Despite innumerable efforts devoted to the X-ray crystallographic analysis of GS-related compounds, except for the few

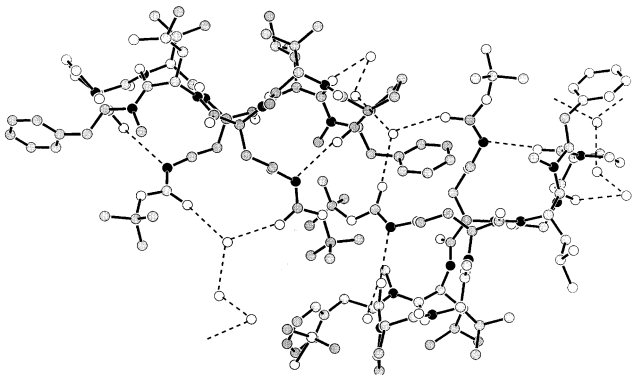

Figure 4. Packing diagram of **1** with possible intermolecular hydrogen bonding shown by broken lines. Hydrogen atoms are omitted. The carbonyl group of the Leu residue and two carbonyl groups of the Boc groups in the Orn side chains of another molecule are connected by a hydrogen-bonding network via three molecules of water. The other water molecule, possibly hydrogen bonded to the carbonyl group of the Pro′ residue, is not shown.

successful examples of the hydrated GS-urea complex,[7d,e] bis-(trichloroacetyl) GS[7f] and bis(*m*-bromobenzoyl) GS,[7f] all other attempted analyses failed without reaching a final structure. The difficulty of making stable single crystals of GS derivatives suitable for crystallography probably arises from the lack of sufficient intermolecular interactions. In the present case, crystallographic analysis has been successfully performed using crystals obtained from DMSO-d_6 solution. It is likely that the solution in the sample tube absorbed water very slowly over a long period of time. Although branched alkyl and Boc groups seem to prohibit good crystal packing, very slow addition of a small amount of water appears to make the intermolecular hydrogen bonding possible, furnishing the single crystals. Once formed, the crystals were stable in the air, and no decomposition was observed. DMSO is usually considered to be the least suitable solvent for recrystallization. However, we consider it possible that a similar method could be applied to the crystallization of other organic compounds which possess low solubility in water.[11]

In the crystal, as expected, **1** adopts the so-called antiparallel pleated β-sheet conformation. Apparently, there seem to be no significant differences between the main-chain conformations of **1** and GS, as well as its derivatives and analogues described in the literature. However, the torsion angle values, ϕ and ψ, differ substantially from those found in crystals and proposed from the theoretical calculations of GS summarized in Table 2. These differences probably indicate that considerable variation in the set of torsion angles is possible without significant change in the overall molecular shape of the GS framework. The pleated sheet is twisted in **1**; that is, the Val and Pro residues are located higher than the Leu and D-Phe residues, as seen from the side view (Figure 2b). The decapeptide rings of the urea complex and the diacyl derivatives of GS are also twisted, but they are twisted in the opposite direction, that is, with the Leu−D-Phe sequences upward and the Pro−Val sequences downward. The difference in the twisting sense between **1** and other derivatives might be related to the difference of the hydrogen-bonding mode described below.

(11) For example: Ohyama, T.; Oku, H.; Hiroki, A.; Maekawa, Y.; Yoshida, M.; Katakai, R. *Biopolymers* **2000**, *54*, 375−378.

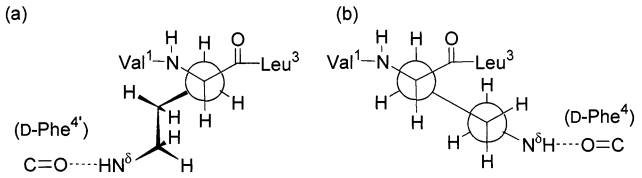

Figure 5. Two hydrogen-bonded conformations of the Orn side chains in GS derivatives. (a) $i \rightarrow i - 3$ hydrogen bonding; $\chi^1 \approx \chi^2 \approx -60$ (g^-), $\chi^3 \approx 180$ (t). (b) $i \rightarrow i + 2$ hydrogen bonding; $\chi^1 \approx \chi^2 \approx \chi^3 \approx 180$ (t).

As for the stereochemistry of the basic side chains in natural GS, elaborate ^{1}H and ^{15}N NMR studies[4i] indicated the presence of hydrogen bonding between the ammonium groups and the carbonyl oxygens of the D-Phe residues which follow the Orn residues, that is, in the $i \rightarrow i + 2$ mode. In the crystal structure of the GS-urea complex,[7d] only one of the two ammonium groups is involved in hydrogen bonding, which is also in the $i \rightarrow i + 2$ mode. Prior to the X-ray study, theoretical calculations predicted the presence of the $i \rightarrow i - 3$ mode hydrogen bonds in GS, that is, between the Orn side chains and the carbonyl groups of the D-Phe residues which precede the Orn residues.[5a] However, subsequent calculations confirmed the stability of the $i \rightarrow i + 2$ hydrogen-bonded structure.[5d] Such hydrogen bonds are also observed in the recently determined crystal structures of the diacyl derivatives, bis-N^δ-(trichloroacetyl) GS and bis-N^δ-(*m*-bromobenzoyl) GS,[7f] which is consistent with the ^{1}H NMR study in solution of the side-chain-protected GS derivatives, including a number of unsymmetrical GS derivatives.[9] However, in the crystal structure of **1** presented in this study, the urethane NHs of the protected side chains are hydrogen bonded to the carbonyl groups of the D-Phe residues which precede the Orn(Boc) residues in the $i \rightarrow i - 3$ mode rather than in the expected $i \rightarrow i + 2$ mode between the N$^\delta$H of the Orn(Boc) and the carbonyl groups of the D-Phe residues which follow the Orn(Boc) residue. Although the difference of the hydrogen-bonding mode could be attributed formally to the presence of the N-methyl groups in **1**, it is difficult to find a rational explanation for this observation.

In solution, a conformation similar to that found in the crystal state is considered to be a major conformation for **1**, or at least one of the main conformations. Because the $i \rightarrow i + 2$ mode conformers are dominant before N-methylation,[9] the Orn side chains must flip from the D-Phe residue on one side to that on the other side during and/or after the methylation. As shown in Table 2 and schematically in Figure 5, the Orn side chains adopt the $t-t-t$ conformation ($\chi^1 \approx \chi^2 \approx \chi^3 \approx 180$) in the $i \rightarrow i + 2$ hydrogen-bonded structure, while the side chains of **1** in the crystal with the $i \rightarrow i - 3$ hydrogen bonding adopt the $g^- - g^- - t$ conformation ($\chi^1 \approx \chi^2 \approx -60$, $\chi^3 \approx 180$). Although the change in the hydrogen-bonding mode requires rotation of about 120° around the C$^\alpha$−C$^\beta$ and C$^\beta$−C$^\gamma$ bonds, the flipping process is considered to be much faster than the methylation reaction, because no appreciable amount of side-chain-methylated products was found in the reaction mixture. To understand the stereochemistry of the substituted Orn side chains in GS, elaborate studies will be necessary, because the methylation was carried out in DMF, while in the present discussion the conformation of the starting material is deduced from the study in DMSO-d_6 solution, and that of the methylated product **1** is based on the crystal structure.

Because of the increasing resistance of bacteria to conventional antibiotics,[12] peptide antibiotics which attack the bacterial

membrane, leading to membrane permeation, are attracting attention as promising antibacterial agents against multidrug-resistant pathogens.[13] While the linear peptide antibiotics of the gramicidin families, gramicidins A−D, are known to form ion channels by adopting helical conformations,[14] the mode of action of GS is not completely established in detail. As an amphiphilic cyclic peptide, GS is generally considered to act as a detergent, resulting in membrane damage and permeability enhancement,[15] for example, via a so-called "carpetlike" mechanism.[13] Tachyplesin I, a disulfide-bridged cyclic heptadecapeptide possessing an antiparallel β-sheet structure, was suggested to act at the lipid membrane by a mechanism essentially similar to that of GS.[16] Antibiotic linear peptides possessing amphiphilic helical structures usually manifest their activity via transmembrane pore formation, that is, via the "barrel-stave" mechanism.[13] Although channel formation was proposed for GS, based on the crystal structure of the GS-urea complex,[7e] it seems unlikely that the antibacterial activity is attributed to the formation of a channel structure in the bacterial membrane, in view of the high antibacterial activity of the N-methyl-group-containing GS analogues such as [MeOrn$^{2,2'}$,D-MePhe$^{4,4'}$]GS, [MeOrn(Me)$^{2,2'}$,D-MePhe$^{4,4'}$]GS, and [MeOrn(Me$_3^+$)$^{2,2'}$,D-MePhe$^{4,4'}$]GS.[8b] Cross-β-sheet hydrogen bonds, which are actually observed in the diacyl GS derivatives,[7f] are considered to play an important role in the stabilization of a channel-type structure in lipid membranes, while such a hydrogen-bonding interaction is impossible in the case of the biologically active N-methylated analogues. A GS analogue lacking hydrophilic amino acid residue was found to possess potent antimicrobial activity against Gram-positive bacteria,[17] and GS-related cyclic peptides with little or no hemolytic activity were reported to exhibit broad spectrum activity toward Gram-positive and Gram-negative bacteria.[18] These results indicated that GS can be considered a versatile lead compound for rational design and the preparation of antibiotic agents effective against multidrug-resistant bacteria.

GS is also an important molecular scaffold for the construction of functional molecules.[19] A variety of functional groups can be introduced on the two amino groups of the Orn side chains, which are located on one side of the β-sheet. For example, 2-pyridyl-moiety-containing groups were attached to GS as metal ion-binding groups,[10,19c] and a dinuclear Zn(II) complex of the tetrakis(2-pyridylmethyl) derivative was found to accelerate the cleavage of the phosphodiester linkage of an RNA model substrate quite effectively.[19c] Although the present study has shed some light on the stereochemistry of the protected side chains in GS derivatives, a more comprehensive understanding of the stereochemical properties of modified side-chain groups of the Orn residues seems essential for the utilization of this β-sheet-based framework as a molecular scaffold for the construction of functional molecules.

Experimental Section

Preparation of 1. To a solution of the bis-Boc derivative [Orn-(Boc)$^{2,2'}$]GS (201 mg) in DMF (3 mL) was added CH_3I (10 mL) and Ag_2O (2.02 g), and the reaction mixture was stirred for 24 h at room temperature. MeOH (20 mL) was added, and the precipitated Ag compounds were filtered off. The filtrate was evaporated, and the residue was purified by SiO_2 chromatography ($CHCl_3$−MeOH) to afford **1** as a colorless solid (218 mg, 89%). Colorless crystals of **1** were obtained from DMSO-d_6 solutions, by absorption of water from the air very slowly through a plastic cap. Mp. 151−152 °C.

Crystallography. A colorless crystal mounted on a fiber was subjected to data collection using an imaging plate diffractometer as summarized in Table 1. The structure was solved by the direct method using the SIR92 program system.[20] The processed structure was refined by using SHELXL-97.[21] Hydrogen atoms were included but were not refined.

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Supporting Information Available: Crystal information file (CIF). This material is available free of charge via the Internet at http://pubs.acs.org.

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