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An Ornithine-Free Gramicidin S Analogue Using Norleucine, Cyclo(Val–Nle–Leu–D-Phe–Pro)₂, Forms Helically Aligned β -Sheets

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The structure of an ornithine (Orn)-free Gramicidin S (GS) analogue, cyclo(Val–Nle–Leu–D-Phe–Pro)₂ (NGS), was studied. Its circular dichroism (CD) spectrum showed that NGS has a structure similar to GS, though the value of $[\theta]$ indicated smaller β -turn and sheet populations. This is probably because the Nle side chain could not form intramolecular hydrogen bonds stabilizing the sheet structure. The chemical shift perturbation of α H and $J_{\text{NH}-\alpha\text{H}}$ were similar in GS and NGS. Three independent NGS molecules formed intramolecular β -sheet structures in crystal. The turn structures of D-Phe-Pro moieties were classed as type II' β -turns, but one part was unclassified. The molecules were arranged in a twisting manner, which resulted in the formation of a helical sheet. Similar structural characteristics were observed previously in a Leu-type, Orn-free GS analogue and in GS trifluoroacetic acid salt.

Key words gramicidin S; ornithine free; helical sheet; type II' β -turn; crystal structure; chemical shift perturbation

Introduction

Gramicidin S (GS) contains the repeating five-residue unit Val–Orn–Leu–D-Phe–Pro (Fig. 1) and exerts antibacterial effects by acting on the cell membrane.¹⁾ The hydrophilic side chains of two ornithine (Orn) residues are located on one side of the peptide ring, and the hydrophobic side chains of Val, Leu and D-Phe on the opposite side give the molecule amphiphilicity.^{2,3)} This physical property contributes to the antibacterial activity of GS and makes it less likely that resistance will develop. For that reason, there is an ongoing effort to develop GS analogues for clinical use. However, before any GS analogue is deemed suitable for use clinically, it must be shown to lack hemolytic activity.^{4,5)}

Many GS analogues have been investigated to better understand their structure and activity relationships.^{6–11)} In 1957, the structure of GS was predicted to be a symmetrical β -sheet containing two β -turns,^{12,13)} and spectroscopic studies supported that prediction.^{14,15)} GS has also been an excellent model for structural studies of β -turns and β -sheets. However, X-ray analysis has long proven difficult. Although the crystal structure of a urea–GS complex (GS-urea) has been reported,^{16,17)} it must be evaluated with caution, since urea is a protein denaturant. In our experiments, crystals of GS·2HCl and acetyl-GS were grown from aqueous alcohol solution, but they were an assembly of microcrystals, which made analysis difficult (the structure of GS·2HCl was solved later). In addition, the purchasable GS·2HCl reagent purified from *Bacillus brevis* (Nagano) was contaminated by several analogues, which also caused the quality of the crystals to be poor. We therefore tried modifying the aminopropyl $-(\text{CH}_2)_3\text{-NH}_2$ group of Orn with trichloroacetyl or *o*-boromobenzyl groups and succeeded in the X-ray analyses.¹⁸⁾ In these cases, the bulky groups induced an energy disadvantage in the Orn side chains and their conformational flexibilities were moderately suppressed. This led the Orn side chains to assume a single conformation favorable for high-resolution structural analysis.

Within unprotected GS structures such as GS trifluoroacetic acid salt (GS·TFA)¹⁹⁾ and GS·2HCl,²⁰⁾ the Orn side chains assume several conformational forms. Their amino groups interact with the disordered solvent molecules, which interferes with convergence of the structural refinement. It appears that some modification of the aminopropyl groups is required to suppress the disordered state. We therefore designed Orn-free

Fig. 1. Chemical Structures of GS and NGS

Orn residues were replaced with norleucine (Nle) in NGS.

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analogues of GS. We previously reported the structure of a Leu-substituted analogue (LGS, cyclo(Val–Leu–Leu–D-Phe–Pro)₂), and the β -turn and sheet structures were discussed.²¹⁾ We have now synthesized a new analogue in which norleucine (Nle) was substituted for Orn in GS. Nle does not branch and has the same bond number as Orn. This analogue has the sequence of cyclo(Val–Nle–Leu–D-Phe–Pro)₂ (NGS) and faithfully reproduces the skeleton of GS (Fig. 1). Because they lack amphiphilicity, these Orn-free analogues exhibit no anti-bacterial activity. However, it is noteworthy that the sequences resemble the cyclic cell-penetrating peptides,²²⁾ which suggests the cell permeability of Orn-free analogues could potentially be developed in further studies. Here, we report the structural characteristics of NGS and compare them to the previously reported structures of GS·2HCl²⁰⁾ and LGS²¹⁾ to assess their potential as research models for studying β -turns and sheets.

Experimental

Materials NGS was synthesized using the liquid-phase method. The *tert*-butoxycarbonyl group (Boc group) was used for protection of the amino groups, and the methyl ester group was used for protection of the carboxy groups. The amino acids were coupled using 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride and 1-hydroxybenzotriazole. A dimethylformamide solution of a decapeptide, H-(D-Phe–Pro–Val–Nle–Leu)₂–OH, was added dropwise to the dichloromethane solution of (benzotriazol-1-yloxy)-tris(dimethylamino)phosphonium hexafluorophosphate and 4-dimethylaminopyridine over the course of several hours, yielding NGS: cyclo(Val–Nle–Leu–D-Phe–Pro)₂. NGS was purified by silica gel column chromatography (*n*-hexane–ethyl acetate system) (MALDI-TOD-MS [M + Na]⁺ C₆₂H₉₄N₁₀O₁₀Na: Calc. 1162.47, Found *m/z* 1162.30). The yield was 18.8%.

Circular Dichroism (CD) Spectrum CD spectra were measured using a JASCO J-820 at a rate of 2 nm/min. The peptides were dissolved in ethanol at a concentration of 0.04 mmol/L. Spectrum data were averaged at 1.0 nm.

NMR The NMR spectrum was measured on a Varian Unity Inova (500 MHz). ¹H-NMR (dimethyl sulfoxide (DMSO)-*d*₆, 298 K) δ : 8.60 (d, 2H, D-Phe NH, *J* = 5.40 Hz), 8.48 (d, 2H, Nle NH, *J* = 9.00 Hz), 8.28 (d, 2H, Leu NH, *J* = 9.00 Hz), 7.14 (d, 2H, Val NH, *J* = 9.00 Hz), 4.66 (dt, 2H, Nle ^αH, *J* = 7.20, 9.00 Hz), 4.49 (q, 2H, Leu ^αH, *J* = 9.00 Hz), 4.42 (m, 2H, D-Phe ^αH), 4.39 (dd, 2H, Val ^αH, *J* = 9.00, 6.60 Hz), 4.32 (t, 2H, Pro ^αH, *J* = 6.60 Hz), 3.55 (dd, 2H, Pro ^βH, *J* = 6.00, 9.00 Hz), 2.89 (d, 2H, D-Phe ^βH, *J* = 8.40 Hz), 2.68 (dd, 2H, Pro ^βH, *J* = 9.00, 17.4 Hz), 2.02 (oct, 2H, Val ^βH, *J* = 6.60 Hz), 1.94 (m, H, Pro ^γH), 1.70 (q, 2H, Nle ^βH, *J* = 7.20 Hz), 1.57 (m, H, Pro ^γH), 1.57 (m, H, Pro ^γH), 1.36 (m, 2H, Leu ^βH), 1.32 (m, 2H, Leu ^γH), 1.26 (m, 2H, Nle ^γH), 1.23 (m, 2H, Nle ^δH), 0.86 (t, 3H, Nle ^αH, *J* = 7.20 Hz), 0.80 (d, 3H, Leu ^δH, *J* = 6.60 Hz), 0.78 (d, 3H, Leu ^δH, *J* = 6.60 Hz), 0.78 (d, 3H, Val ^γH, *J* = 6.60 Hz), 0.76 (d, 3H, Val ^γH, *J* = 6.60 Hz), 4.49 (dd, 2H, Val ^αH, *J* = 9.00, 6.60 Hz).

X-Ray Diffraction The crystals of NGS were grown from aqueous isopropanol solution by a traditional vapor diffusion method (2–5 mg NGS/0.5 mL 60% isopropanol). The diffraction intensities were attenuated in the high-resolution range, and the *R*_{INT} of the 0.95–0.90 Å shell was more than 0.2. The data for the structural refinement were therefore limited to 0.92 Å resolution. Hydrogen atoms in the peptides were cal-

culated from their geometrically ideal positions, and hydrogen atoms in O–H groups were picked up from the Fourier map considering hydrogen-bonding networks. Three peptide molecules were found in the asymmetric unit (*A*, *B* and *C*). The phenyl group of D-Phe4 in molecule *C* (D-Phe4C) was disordered into two sites. The split position of the D-Phe4C side chain induced a disordered state among the solvent molecules. Five isopropanol (iPrOH) molecules were found (*D*–*H*), and molecules *F*–*H* were in a disordered state; the occupancies of *F*, *G* and *H* were 0.488, 0.552 and 0.522, respectively. The geometrical restrictions using DFIX and DANG instructions were adopted for iPrOH molecules in the refinement. Seven water molecules were found (O1W–O7W), and O5W–O7W were in a disordered state; the occupancies of O5W, O6W

Fig. 2. CD Spectra of GS Analogues

Data for LGS and GS·2HCl are from a previous study.²¹⁾ Peptides were dissolved in ethanol at 4×10^{-5} mol/L.

Fig. 3. Chemical Shift Perturbations of α H and $J_{\text{NH-}\alpha\text{H}}$ of the Amide Protons in NGS and GS

(a) $\Delta\delta$ s were calculated as the differences between the δ values in NGS and the random-coil NMR parameters (Bundi & Wuthrich).²⁶⁾ (b) Coupling constants between CONH and α H ($J_{\text{NH-}\alpha\text{H}}$). The $\Delta\delta$ and *J* values for GS are from an earlier study (Tamaki *et al.*).²⁵⁾

Fig. 4. ORTEP Drawing of Three Independent Molecules (*A*, *B* and *C*)

Residues of *A* and heteroatoms are labeled. Blue and red ellipsoids represent nitrogen and oxygen atoms, respectively. Dotted lines show intermolecular hydrogen bonds. The D-Phe4 side chain in molecule *C* (D-Phe4C) is disordered into two parts. Displacement ellipsoids are drawn at the 50% probability level. (Color figure can be accessed in the online version.)

and O7W were 0.448, 0.552 and 0.552, respectively. The structure was solved using SHELXT²³⁾ and refined with SHELXL.²⁴⁾ Triclinic, *P*1, $3(\text{C}_{62}\text{H}_{94}\text{N}_{10}\text{O}_{10}) \cdot 3.552(\text{C}_3\text{H}_8\text{O}) \cdot 5.552\text{H}_2\text{O}$, *M*_r = 3731.9, *a* = 14.8395(3) Å, *b* = 14.6078(4) Å, *c* = 27.7844(7) Å, α = 92.678(2)°, β = 100.951(2)°, γ = 115.486(2)°, *V* = 5281.4(2) Å³, *T* = 93 K, No. of reflections (obs) = 71057, *R*_{INT} = 0.0509, No. of reflections (*I* > 2σ(*I*)) = 23205, *R*₁ = 0.0885, *wR* = 0.2449, Goodness of fit = 1.035, Δρ_{max/min} = 0.841/−0.633 e Å^{−3}. CCDC2093899.

Results

CD Spectra The CD spectra for GS·2HCl, LGS and NGS were measured in ethanol (Fig. 2). Negative Cotton bands were observed at 200–240 nm, and the patterns of the three spectra were similar. The [θ]₂₁₅ of NGS was 50% for GS·2HCl and 75% for LGS, which indicates a decrease in the population of β-turn and sheet structures.²¹⁾ The amino group in the Orn side chain was able to form an intramolecular hydrogen bond with the carbonyl oxygen of D-Phe, stabilizing the β-sheet structure of GS.¹⁸⁾ This likely explains the reduced population of β-turn and sheet structures in Orn-free analogues. There is a noteworthy difference between the LGS and NGS spectra, which is presumed to be due to steric hindrance caused by the presence or absence of side chain branching, but the actual relation is unknown.

¹H-NMR Chemical shift perturbations of αH (CSP) and coupling constants of the amide protons (*J*_{NH–αH}) reflect environmental changes around the protons, and their calculation is a simple experimental technique for comparing solution structures. This method was adapted to compare NGS with previously reported GS data²⁵⁾ (Fig. 3). CSP was observed as Δδs calculated as the differences between the δ values for NGS and the random-coil NMR parameters.²⁶⁾ The Δδ values for Val, Nle, Leu were positive, while those for D-Phe and Pro were negative. Although there were some differences in the

Table 1. Selected Hydrogen Bond Geometry (°, Å)

D	A	<D–H...A	D...A* symm.
Intramolecular			
N31A	O62A	168	2.84
N61A	O32A	159	3.02
N81A	O12A	155	2.90
N11B	O82B	167	3.18†
N31B	O62B	167	2.88
N61B	O32B	161	3.04
N81B	O12B	160	2.96
N11C	O82C	155	2.98
N31C	O62C	173	2.80
N61C	O32C	154	2.90
N81C	O12C	172	2.91
Intermolecular			
N21A	O52C	163	2.96 (x, y, z−1)
N41A	O72C	168	3.05 (x, y, z−1)
N71A	O22B	155	2.82
N21B	O52A	143	2.85
N71B	O02C	161	2.82
N21C	O72B	170	2.97
N71C	O22A	172	2.82 (x, y, z+1)

*The esd's of the D...A distance are approximately 0.009–0.02 Å. †Somewhat long for a hydrogen bond, but included in the table.

magnitudes of Δδ, the shift patterns were consistent between GS and NGS. When *J*_{NH–αH} were compared, no significant difference was observed between GS and NGS. These results suggest that the environments around the αH and amide protons are similar between NGS and GS, which indicates they have similar backbone structures.

Molecular Structures Three independent molecules (*A*, *B* and *C*) were present in the asymmetric unit, and all formed β-sheet structures with intramolecular hydrogen bonds (Fig. 4). In molecule *B*, the N11B...O82B distance (=3.18 Å) was

slightly longer than the standard distance (Table 1). Similar hydrogen bond geometry was detected in the structure of GS·2HCl.²⁰ The D-Phe4 side chain in molecule *C* (D-Phe4C) was disordered to two sites. In molecules *B* and *C*, the phenyl rings of D-Phe overhang along the long axis of the peptide ring. By contrast, the phenyl ring of D-Phe9 in molecule *A* (D-Phe9A) protruded laterally with respect to the long axis, and the carbonyl oxygen atom (O82A) of the preceding Leu8A was sprung out.

Backbone and Nle Side Chain Conformations To compare the structures of the three molecules, molecular fitting of the peptide backbone chains was performed using PROFIT.²⁷ At that time, molecule *A* of GS·2HCl was also fitted (Fig. 5). The O82A atom of D-Phe8A was differently oriented, though the RMSDs for N, C α , C and O atoms were as small as 0.545–0.625 Å, which indicates that the four GS backbone structures are fairly similar to one another. When all non-

Fig. 5. Superimposition of Backbones among Three Molecules of NGS and GS·2HCl

Black bonds show GS·2HCl (molecule *A*). (Color figure can be accessed in the online version.)

hydrogen atoms were plotted, it could be seen that the side chains have various conformations (Supplementary Materials, Fig. S1). When focusing on the Orn side chains, two forms (e-form and f-form) were observed in the GS·2HCl crystal. In the e-form, the Orn side chains extended to the outer solvent region (Fig. 6a), and the amino groups in the Orn side chains interacted with solvent molecules. As a result, the conformation of the Orn side chain in the e-form was strongly affected by the solvent state. This e-form was previously found only once, in the GS·urea complex.^{16,17} In the f-form, the Orn side chains are folded and form intramolecular hydrogen bonds (Fig. 6b), and two modes of donor-acceptor combination ($i \rightarrow i+2$ or $i \rightarrow i-3$) have been reported.²⁸ It appears that the f-form stabilizes the β -sheet structure and has often been observed in the crystal structures of GS analogues. In NGS molecules, the e-forms were observed for molecules *A* and *B*, though the Nle7 side chain of molecule *C* (Nle7C) was close to the f-form (Fig. 6e). The f-form was not detected in the related LGS analogue,²¹ and the presence of the f-form shows that the NGS structure reflects the characteristics of the parental GS structure.

Type II' β -Turn Table 2 shows the torsion angles ϕ (N–C α bond) and φ (C α –CO bond) for comparison of the conformations of the main chains. The ω angles (CO–NH) are omitted because all amide bonds were *trans*. The β -turn unit was composed of four residues with a NH...O= hydrogen bond ($i+3 \rightarrow i$), and the D-Phe–Pro moiety was located at the $i+1$ and $i+2$ positions and defined the β -turn type. The values of (φ_4, ψ_4) and (φ_9, ψ_9) were distributed around ($60^\circ, -120^\circ$), while (φ_5, ψ_5) and (φ_{10}, ψ_{10}) were distributed around ($-80^\circ, 0^\circ$), which indicates a type II' β -turn. Molecule *A* is exceptional in that the carbonyl group of Leu8A (O82A) was flipped to the outside, and φ_8 differed from the other molecules. This distortion affects the turn structure of D-Phe9–Pro10, which is unclassified as any type of β -turn. A similar unclassified turn was also observed in LGS but not in Orn-containing analogues, which form intramolecular hydrogen bonds.

Fig. 6. Conformations of the Orn and Nle Side Chains

(a) In molecule *A* of GS·2HCl, the Orn side chains extend perpendicular to the peptide ring and interact with solvent molecules (e-form). (b) In the molecule *B* of GS·2HCl, the Orn side chains are folded and form intramolecular hydrogen bonds. (c) and (d) Molecules *A* and *B* of NGS. Nle side chains assume a conformation similar to the e-form. (e) The conformation of the Nle7 side chain in molecule *C* (Nle7C) is close to the f-form. One part of disordered D-Phe4C side chain was plotted by crosses. (Color figure can be accessed in the online version.)

Table 2. Torsion Angles of the Peptide Backbones ($^\circ$)

Residue	Type	<i>A</i>	<i>B</i>	<i>C</i>
Val1	ϕ_1	−139.3(8)	−111.3(9)	−83(1)
	φ_1	164.5(8)	144.5(7)	125(1)
Nle2	ϕ_2	−114(1)	−77(1)	−100(1)
	φ_2	130.7(9)	133.6(8)	118(1)
Leu3	ϕ_3	−119.3(9)	−144.2(8)	−120(1)
	φ_3	104.3(8)	110.0(8)	92(1)
D-Phe4	ϕ_4	58.4(9)	57(1)	62(1)
	φ_4	−135.5(7)	−132.2(9)	−128(1)
Pro5	ϕ_5	−88.8(9)	−100(1)	−93(1)
	φ_5	8(1)	11(1)	15(1)
Val6	ϕ_6	−93.1(9)	−116.5(9)	−108(1)
	φ_6	116.1(8)	178.7(8)	127.1(9)
Nle7	ϕ_7	−83.6(9)	−122(1)	−109.2(9)
	φ_7	110.5(7)	129(1)	121.4(9)
Leu8	ϕ_8	−88.7(9)	−124(1)	−113.4(9)
	φ_8	−5(1)	115.0(9)	96.2(9)
D-Phe9	ϕ_9	131.0(8)	55(1)	54(1)
	φ_9	−82.1(9)	−129.4(8)	−133.8(8)
Pro10	ϕ_{10}	−86(1)	−92(1)	−81(1)
	φ_{10}	−13(1)	0(1)	−6(1)

Table 3. Puckering Parameters of the Pyrrolidine Ring

	Q2*	$\phi 2^*$	χ^1	χ^2	χ^3	χ^4	θ^{**}	Twist [†]
Pro5A	0.38(1)	76(2)	36.1(9)	−37.9(9)	25.0(9)	−2.2(9)	−21.3(8)	52.3
Pro10A	0.39(1)	89(2)	33(1)	−41(1)	32(1)	−12(1)	−13(1)	
Pro5B	0.40(1)	67(2)	39(1)	−38(1)	21(1)	4(1)	−27(1)	75.7
Pro10B	0.39(1)	89(2)	41(1)	−40(1)	22.9(9)	3.1(9)	−27(1)	
Pro5C	0.39(1)	64(2)	39(1)	−39(1)	18(1)	7(1)	−29(1)	78.6
Pro10C	0.36(1)	82(2)	33(1)	−37(1)	26(1)	−6(1)	−17(1)	

*The Q2 and $\phi 2$ are the amplitude and phase of the puckering defined by Cremer and Pople.²⁹⁾ **The θ angle indicates the unique pyrrolidine angle of $C\gamma-N-C\alpha-C\beta$.³⁰⁾ †The angles between the two least-squared planes at turn positions: [N(d-Phe4), Ca (d-Phe4), C(d-Phe4), N(Pro5), Ca (Pro5) and C(Pro5)] and [N(d-Phe9), Ca (d-Phe9), C(d-Phe9), N(Pro10), Ca (Pro10) and C(Pro10)].

Fig. 7. Hydrogen Bond Networks Relating with Molecules A and B

Red balls show water molecules. Dotted arrows show hydrogen bonds from donor to acceptor. Gray labels are symmetry-translated atoms. Molecules D and E are solvated iPrOH molecules. (Color figure can be accessed in the online version.)

Fig. 8. Hydrogen Bond Networks Relating with Molecules B and C

Red balls show water molecules. Dotted arrows show hydrogen bonds from donor to acceptor. Gray labels are symmetry-translated atoms. Molecules D and E are solvated iPrOH molecules. (Color figure can be accessed in the online version.)

Peptide Twisting and Puckering of the Pyrrolidine Ring

The peptide rings in NGS are twisted to the right along its long axis. The twisting indexes were calculated as angles between the two least-squared planes of the d-Phe-Pro moieties ("twist" in Table 3). These planes were composed of N(d-Phe), Ca (d-Phe), C(d-Phe), N(Pro), Ca (Pro) and C(Pro). The angles were 52.3–78.6° in NGS and were slightly larger than in the other analogues (Supplementary Materials, Table S2). Pro has a pyrrolidine ring that is able to pucker in several forms. Table 3 shows the parameters of the 5-membered rings. Q2 and $\phi 2$ are the amplitude and phase of the puckering defined by Cremer and Pople.²⁹⁾ The χ angles indicate the torsion angles of the Pro side chains, and the θ angle indicates the unique pyrrolidine angle $C\gamma-N-C\alpha-C\beta$.³⁰⁾ The χ angles indicate that

Fig. 9. Hydrogen Bond Network in the Disordered Region

The rotation of the phenyl ring of d-Phe7C changes the space size of this region, and two disordered states (Part 1 and Part 2) are observed. In the Part 1 state (upper), the solvent molecule F and water O5W are located. Alternatively, the solvent molecules G and H, water O6W and O7W appear in the Part 2 state (lower). (Color figure can be accessed in the online version.)

the pyrrolidine puckering assumes the $C\gamma$ -endo form. Similar puckered forms were observed in the other GS analogues. Notably, Q2 in GS·Urea, which is a complex containing a denaturant, is smaller than in the other analogues. This is interesting, considering that the Pro monomer is in the $C\gamma$ -exo form.³¹⁾ The accesses between the phenyl group of the preceding d-Phe and the $C\gamma$ atom of Pro were observed in 4 out of 6 residues contributing in a single puckered form.

Intermolecular Interactions The least-squared planes containing ten Ca atoms (LSP) were calculated for three molecules, and the LSP...LSP angles between molecules A...B, B...C and C...A were 106°, 114° and 137°, respectively. Because molecules A and B form a relatively large acute angle of 74° (the LSP...LSP angle = 106°), two peptide-peptide hydrogen bonds, N21B...O52A and N71A...O22B, were formed (Table 1 and Fig. 7), and the solvent molecules O2W and iPrOH mediated a long bridge, O72A...O2W...O1D...O42B, which stabilized the intermolecular β -sheet structures (Supplementary Materials, Table S1). At Leu8A, the flipped carbonyl oxygen O82A interacted with O4W bridging the translated

Fig. 10. Packing Diagram in Crystal

Side chains are omitted for clarity. The disordered regions are shaded. NGS molecules are twisting forming a helical β -sheet (upper). Solvent molecules located between the peptides support this sheet structure. A diagram summarizing the arrangement is below. The LSP...LSP angles between molecules $A...B$, $B...C$ and $C...A$ are 106° , 114° and 137° , respectively. (Color figure can be accessed in the online version.)

O42A. In addition, A also interacted with the translated C , forming three intermolecular hydrogen bonds: N21A...O52C, N41A...O72C and N71C...O22A. The acute angle of 43° between molecules A and C (the LSP...LSP angle = 137°) was smaller than the other adjacent combinations, making formation of peptide-peptide hydrogen bonds easier. Moreover, O1W mediated interaction between molecules A and translated C : N91C...O1W...O02A and O72C...O1W...O02A (Table S1).

Two hydrogen bonds are formed between molecules B and C (Fig. 8), N21C...O72C and N71B...O02C, and solvent mediated interactions are found in the disordered region. The disordered state was divided into two states based on the position of the D-Phe4C phenyl ring (Fig. 9). In one state (Part 1), the phenyl ring was close to molecule B (Fig. 10 upper), and the located O5W and iPrOHF bridged molecules B and C : N91B...O5W...O22C. In the other state (Part 2), the phenyl group was moved back, and O6W, O7W, iPrOHG and iPrOHH appear (Fig. 10 lower). O7W bridged molecules B and C , N91B...O7W...O22C, and O6W and O1F linked to the other translated molecules.

Helical Structures The packing diagram for NGS is shown in Fig. 10. The three molecules were helically arranged along the c -axis forming β -sheets. Solvent molecules were located among the peptide molecules and bridged the helical sheet structure. The shadowed areas are the disordered solvent regions. A similar helical β -sheet was observed in LGS and another type of helical sheet was also found in GS-TFA, but not GS-2HCl (the LSP...LSP angle = 49° ²⁰). When NGS molecules were arranged side-by-side, the intermolecular contacts between the side chains defined the rotation of the peptide molecules. The interactions with solvent molecules contributed to the rotation angle.

Conclusion

The number of bonds in the Nle side chain is equal to that in Orn, and the skeleton of the entire NGS accurately reflects that of GS. The CD spectrum shows the β -turns and sheets of NGS in solution, though their population is smaller than in

GS. From the measurements of CSP and $J_{(\alpha\text{H}-\text{NH})}$, it is inferred that the environments of the $\text{C}\alpha$ and amide protons are homogeneous between GS and NGS solutions, where the peptides have similar backbone structures. X-ray structural analyses show that three molecules of NGS form similar β -sheet structures with a type II' β -turn, though one part of the turn in molecule A is distorted. In addition, the N11B...O82B distance is somewhat longer than the standard hydrogen bond. The puckering of the pyrroline rings was all C_γ -endo, which has been observed in all GS structures revealed so far. The Nle side chains take the e -form, and the Nle7C side chain is exceptionally close to the f -form. The three molecules are arranged in a twisting manner and form a helical β -sheet structure stabilized by intramolecular hydrogen bonds and solvent bridging. These characteristics of the NGS structure reflect those of parent GS-2HCl and other analogues, which provides a good scaffold model for studying β -turns and sheets.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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