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Carbohydrate Recognition of Gramicidin S Analogues in Aqueous Medium

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Abstract—We have designed and synthesized of carbohydrate-binding peptides, gramicidin S analogues. Asn/Asp/Gln and Trp residues in the peptides were employed as the binding sites for carbohydrates by hydrogen-bonding interaction and the creation units for hydrophobic pocket to promote the interaction, respectively. The data of fluorescence spectroscopy and affinity column chromatography indicated that the peptides possessed the binding ability for some carbohydrates in aqueous medium. As a result of ^{1}H NMR study, nuclear Overhauser effects between aromatic side chains of a peptide, [Gln$^{1,1'}$,Trp$^{3,3'}$]-gramisidin S and mannose were observed, indicating that the interaction of the peptide with the sugar occurred in the hydrophobic environment formed by Trp and Phe residues.

It is well known that carbohydrates on cell surfaces play important roles in several biological events occurring between cells such as immune response, viral infection, and metastasis of tumor cells.[1] The regulation of carbohydrate recognition using artificial molecules is necessary to develop novel drugs, which can control such biological functions. From this viewpoint, much attention has been attracted to the molecular design of carbohydrate-binding molecules useful in aqueous medium. Shinkai and coworkers have demonstrated that the molecules bearing boronic acids are of convenience to touch carbohydrates in aqueous solution, in which the covalent linkages can be formed between carbohydrate and the boronic acid.[2,3] As carbohydrate recognition processes, however, are mostly mediated by polypeptides in biological systems, it is expected to construct several lectin-mimicking molecules using the peptide-based system. Recently, peptide-based molecules, which can bind to ganglioside GM1, were reported by Sato et al.[4] They obtained the peptides from a phage-displayed pentadecapeptide library. In this study, we tried de novo design of carbohydrate-binding molecules using cyclic peptides containing Trp and Asn/Asp/Gln residues, gramicidin S analogues, and report their interaction with carbohydrates assessed by fluorescence spectroscopy, affinity column chromatography and nulear magnetic resonance.

Here, considering the structures of some lectins, carbohydrate-binding proteins,[5] we state three points necessary for the design of the molecule as follows, that is, rigid backbone, hydrogen-bonding sites between the peptide and carbohydrate, and hydrophobic pocket to promote hydrogen-bonding effectively. From these viewpoints, we employed an antibacterial peptide, gramicidin S (GS), produced by *Bacillus brevis*,[6] as a backbone (Fig. 1). GS is a cyclic peptide consisting of 10 amino acid residues and has β-sheet structure in the molecule, in which the directions of the side chains are comparatively restricted. Therefore, we put up GS as a candidate for the rigid backbone of our target molecule. To form the hydrogen-bonding between the peptide and carbohydrate, Asx (Asp or Asn) and Gln residues were substituted for Val residues in GS. The hydrophobic pocket to exert the effect of hydrogen-bonding was prepared by substituting Trp residues for Leu residues in GS. To find optimum peptide structure for binding of carbohydrates, we designed and synthesized three kinds of GS analogues, [Asn$^{1,1'}$, Trp$^{3,3'}$]-GS, [Asn^1, Asp$^{1'}$, Trp$^{3,3'}$]-GS, [Asp$^{1,1'}$, Trp$^{3,3'}$]-GS and [Gln$^{1,1'}$, Trp$^{3,3'}$]-GS,

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Gramicidin S (GS)	cyclo(Pro-Val-Orn-Leu-D-Phe)$_2$
NNW-GS	cyclo(Pro-Asn-Orn-Trp-D-Phe)$_2$
DNW-GS	cyclo(Pro-Asn-Orn-Trp-D-Phe--Pro-Asp-Orn-Trp-D-Phe)
DDW-GS	cyclo(Pro-Asp-Orn-Trp-D-Phe)$_2$
QQW-GS	cyclo(Pro-Gln-Orn-Trp-D-Phe)$_2$

[Orn: ornithine]

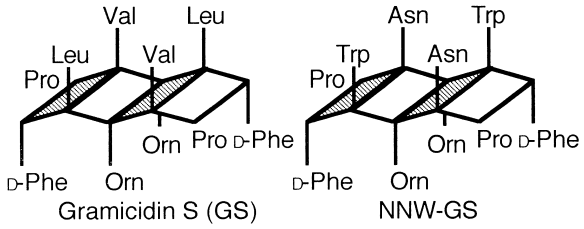

Figure 1. Structures of gramicidin S and its analogues.

abbreviated as NNW-GS, DNW-GS, DDW-GS and QQW-GS, respectively (Fig. 1).

These GS analogues were synthesized by Fmoc solid-phase synthesis on Fmoc-Asp(OAll)-Rink Amide resin or -Wang resin (NOVA Biochem). After elongation of peptide chain, allyl ester (OAll) was removed by Pd(0) catalyzed allyl transfer.[7] Cyclization of a peptide attached via the side chain of an Asp residue was performed on the resins using HBTU [2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] and HOBt (1-hydroxybenzotriazol).[8] After deprotection and cleavage from the resins, the peptides were purified by reversed phase HPLC, and then identified by FAB-MS.[9]

The interaction of the peptides with carbohydrates was assessed by measuring changes in fluorescence intensity of Trp residues in the peptides upon addition of carbohydrates. In the case of NNW-GS, a maximum of fluorescence intensity at 352 nm was reduced to 97 and 94% of original fluorescence intensity without peak shift by addition of fructose and lactulose, respectively (Fig. 2). As can be seen in Table 1, similar results were obtained in the cases of NDW-GS, DDW-GS and QQW-GS. Although we can not well explain the reduction of the fluorescence intensity, it is reasonable to suppose that the addition of the carbohydrates leads to some changes in microenvironments of Trp residues of the peptides. Furthermore, the change in fluorescence intensity was complete within 1 min as the results of time course measurements show (Fig. 3). On the other hand, no significant changes in the spectra of all analogues were observed in the cases of the addition of glucose, and galactose (Table 1).

In order to confirm the direct interaction of GS analogues with the carbohydrates, the analogues were applied to a lactulose-immobilized affinity column. As shown in Fig. 4, elution of NNW-GS and QQW-GS was significantly delayed compared with that of DDW-GS, indicating that binding abilities of NNW-GS and QQW-GS were stronger than that of DDW-GS although NNW-GS and QQW-GS did not adsorb on the column completely. These results suggest that the existence of an amide group of amino acid residues in NNW-GS and QQW-GS allow interactions for recognition of lactulose.

The binding ratios of the analogues and carbohydrates were evaluated by measuring the changes in the fluorescence intensities at various concentrations of the carbohydrates (Fig. 5). From the results of log plots (Fig. 5,

Table 1. Change in fluorescence intensity of GS analogues by addition of carbohydrates

Carbohydrate	Change in fluorescence intensity at 352 nm (%)			
	NNW-GS	DNW-GS	DDW-GS	QQW-GS
Lactulose	93.6	92.4	97.4	93.1
Fructose	96.8	95.4	95.1	97.5
Glucose	101	100	99.6	100
Galactose	101	100	101	101

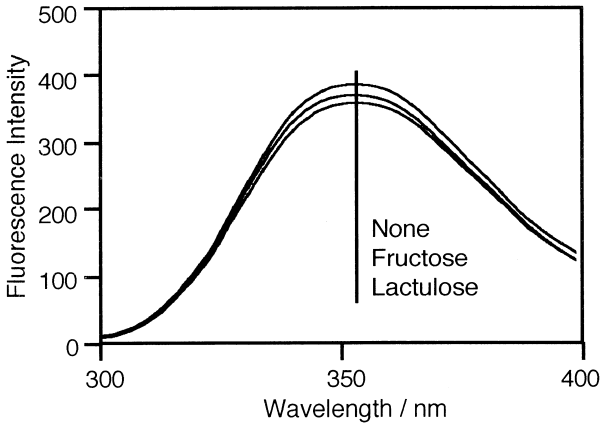

Figure 2. Change in fluorescence spectrum of NNW-GS by addition of lactulose and fructose. NNW-GS and carbohydrates were mixed in 20 mM Tris–HCl buffer (pH 7.4) at final concentrations of 30 μM and 50 mM, respectively. After incubation for 10 min at 37 °C, fluorescence spectra (excited at 280 nm) were measured.

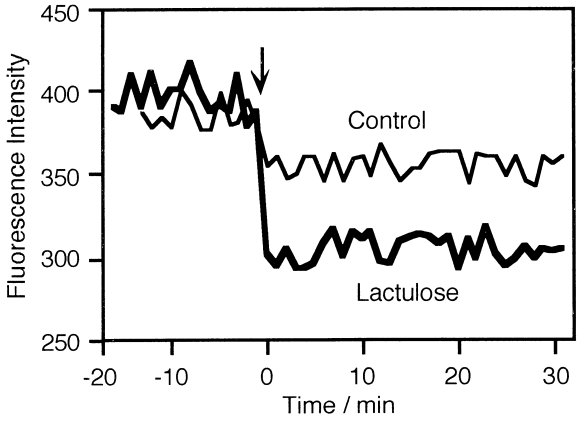

Figure 3. Time course of change in fluorescence intensity of DNW-GS by addition of lactulose. One hundred microliters of 1 M lactulose aqueous solution or the same volume of water was added to 1 mL of 50 μM DNW-GS in 20 mM Tris–HCl buffer (pH 7.4). The fluorescence intensities were measured at an interval of 1 min. Arrow indicates the point in time of the addition of the lactulose solution or water.

inset),[10] it was found that the binding between the GS analogues and carbohydrates occurred at a molar ratio of ca. 1 (Table 2). However, in these plots, the concentration of the GS analogues is too high to ignore, comparing with that of the carbohydrates. Consequently, in order to obtain the precise dissociation constants, we recalculated the binding parameters using a secondary equation on the assumption that the binding ratio between the analogues and carbohydrates is 1.0.[11] As shown in Table 2, NNW-GS possesses stronger binding ability for lactulose with K_d of 0.20 mM, when compared to fructose with K_d of 1.5 mM. The similar tendency was observed in the cases of DNW-GS. These results indicate that both NNW-GS and DNW-GS prefer the structure of lactulose to that of fructose. In the case of DDW-GS, the dissociation constant for lactulose could not be determined, since the change in fluorescence intensity by addition of fructose was smaller than in the case of lactulose as shown in Table 1. Actually, little interaction of DDW-GS with lactulose was observed in the affinity chromatography. Two carboxyl groups in DDW-GS would be disadvantagous to form hydrogen-bonding with the hydroxyl groups of fructose. QQW-GS interestingly showed strong affinity to lactulose ($K_d = 30$ μM). The binding pocket made by glutamine residues would prefer the structure of lactulose (Galβ1-4Fru).

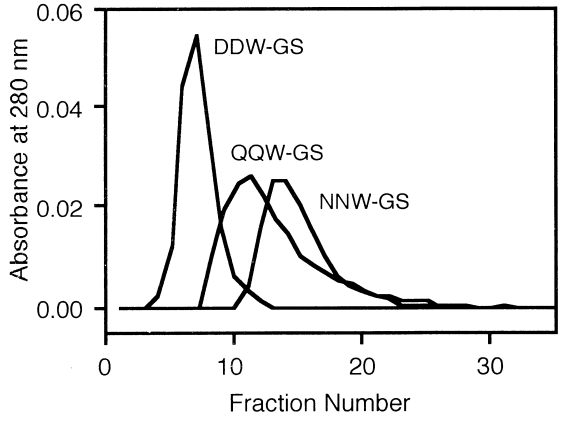

Figure 4. Elution profiles of NNW-GS from a lactulose-immobilized affinity column. Lactulose was immobilized to Cellulofine (Seikagaku Corporation, Tokyo, Japan) using divinyl sulfone as a cross-linker.[12] NNW-GSk, DDW-GS or QQW-GS (70 μg in 200 μL of elution buffer) were applied to the affinity columns (ϕ14×50 mm). The column was eluted with 20 mM Tris–HCl buffer (pH 7.4) at flow rate of 0.5 drop/s, and then 1 mL fractions were collected. Peptide concentration of each fraction was decided by absorbance at 280 nm.

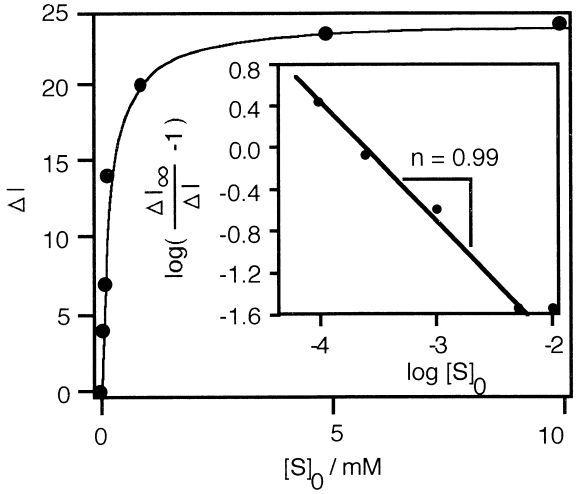

Figure 5. Changes in the fluorescence intensities of NNW-GS at various concentrations of lactulose. Inset shows the log-plot by the equation in ref 10.

In order to obtain detailed information concerning the binding site of peptide with carbohydrate, a two-dimensional ^{1}H NMR study was performed. In this experiment, mannose and QQW-GS were employed because mannose, a monosaccharide, makes assignment of the protons easy and QQW-GS showed high affinity to mannose ($K_d = 1.5\times10^{-4}$ M). From rotating frame Overhauser effect spectroscopy (ROESY) NMR experiments (operating at 600 MHz, mixing time = 100 ms) of 2 mM QQW-GS and mannose solution in ^2H_2O, it was found that intermolecular ROE cross peaks between two aromatic side chains (Trp Hϵ3, Hζ2 and Phe Hϵ1, Hϵ2) and mannose (Hα2, Hα3, Hα6) were observed, suggesting that the aromatic rings of Trp and Phe residues form hydrophobic interactions with mannose (Fig. 6). In addition, significant ^{1}H chemical shift perturbation of Trp Hα and Pro Hδ with higher field shift and Orn Hα with lower field shift were observed by addition of mannose. These results are consistent with changes in fluorescence intensity of the GS-analogues in this work. Further structural refinements of intermolecular arrangement of QQW-GS and mannose or lactulose based on NMR derived constraints are in progress.

In this report, we have introduced novel cyclic peptides, which interact with some neutral carbohydrates. At this stage, we can not explain the selectivity against carbohydrates, that is, why the fluorescence intensity of NNW-GS was reduced by addition of lactulose, but not glucose. The answer will be cleared by preparing several derivatives of GS and detailed structural analysis of the derivatives. On the other hand, from the standpoint of extensive application, peptide has advantages in the design of various molecules by altering the sequence and introducing artificial amino acids, expecting that the carbohydrate-binding molecules based on the peptide allow us to design various molecules in response to their purpose without restriction.

Table 2. Binding ratios and dissociation constants of the GS analogues and carbohydrates

Peptide	Ratio (n)		Dissociation contant (K_d) (M)	
	Lactulose	Fructose	Lactulose	Fructose
NNW-GS	0.99	0.85	2.0×10^{-4}	1.5×10^{-3}
DNW-GS	0.80	0.86	1.8×10^{-4}	4.7×10^{-3}
DDW-GS	n.d.	0.97	n.d.	1.5×10^{-3}
QQW-GS	1.2	n.d.	3.0×10^{-5}	n.d.

n.d., precise data could not be determined due to slight changes in the fluorescence intensities. The GS analogues (final concn 30 μM) were added to solutions of various concentrations of the carbohydrates (0.1–10 mM) in 20 mM Tris–HCl buffer (pH 7.4).

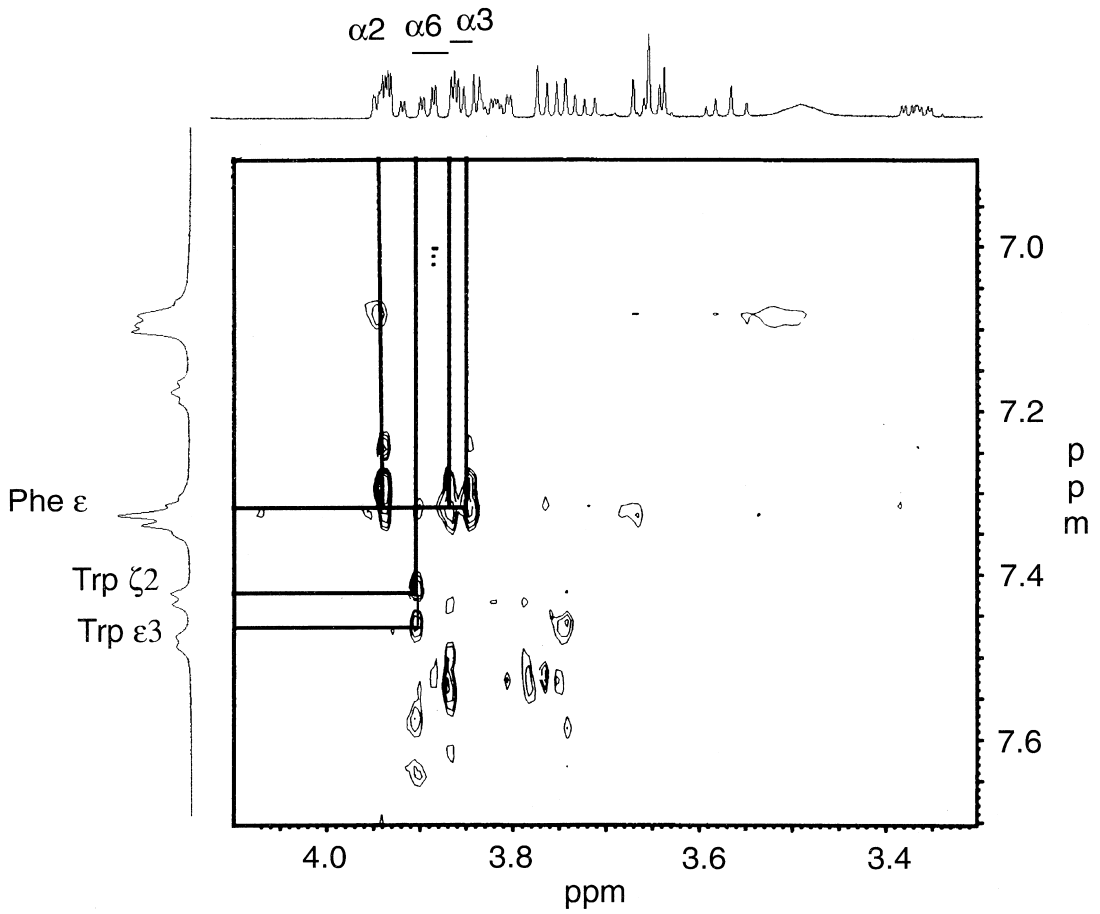

Figure 6. Expanded ROESY spectrum of 2 mM solution of QQW-GS and mannose operating at 600 MHz with 1 K and 512 points. Mixing time, temperature and accumulation time were 100 ms, 30 °C and 120 scans. DQF-COSY and TOCSY NMR spectra of the solution were observed to complete a sequential assignment.

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9. FAB-MS (*m*/*z*): $[M+H]^+ = 1317$, 1318 and 1319 for NNW-GS, NDW-GS and DDW-GS, respectively.
10. $\log(\Delta I_{max}/\Delta I - 1) = \log K_d - n\log[S]_0$, ΔI_{max} = maximum change in intensity, ΔI = change in intensity, K_d = dissociation constant, n = binding ratio, $[S]_0$ = initial concentration of carbohydrate.
11. $\Delta I = \left(\Delta I_{max}\left(([P]_0+[S]_0+K_d) \pm \left(([P]_0+[S]_0+K_d)^2 - 4[P]_0[S]_0\right)^{1/2}\right)\right)/2[P]_0$, ΔI_{max} = maximum change in intensity, ΔI = change in intensity, K_d = dissociation constant, $[S]_0$ = initial concentration of carbohydrate, $[P]_0$ = initial concentration of peptide.
12. Teichberg, V. I.; Aberdam, D.; Erez, U.; Pinelli, E. *J. Biol. Chem*. **1988**, *263*, 14086.