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Syntheses of low-hemolytic antimicrobial gratisin peptides

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ABSTRACT

Antibiotic and hemolytic activities of gratisin (GR), cyclo(-Val^1-Orn^2-Leu^3-D-Phe^4-Pro^5-D-Tyr^6-)$_2$, and fifteen GR analogues, which have various D-amino acid residues in place of D-Tyr$^{6,6'}$ residues, were examined. Among them, [D-Orn$^{6,6'}$]-GR, [D-Lys$^{6,6'}$]-GR and [D-Arg$^{6,6'}$]-GR showed the strong activity against both Gram-positive and Gram-negative bacteria. In addition, the antibiotics showed significantly reduced toxicity against human blood cells compared with gramicidin S, cyclo(-Val^1-Orn^2-Leu^3-D-Phe^4-Pro^5-)$_2$.
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Gramicidin S (GS)[1,2], cyclo(-Val^1-Orn^2-Leu^3-D-Phe^4-Pro^5-)$_2$, tyrocidine A (TA)[1,3], cyclo(-Val^1-Orn^2-Leu^3-D-Phe^4-Pro^5-Phel^6-D-Phe^7-Asn^8-Gln^9-Tyr^{10}-) and gratisin (GR)[1b,4] cyclo(-Val^1-Orn^2-Leu^3-D-Phe^4-Pro^5-D-Tyr^6-)$_2$, are potent cyclopeptide antibiotics with the amphiphilic β-sheet conformation. In view of widespread antibiotic resistance that has become a serious threat to public health,[5] the amphiphilic antibiotics are attractive targets for drug discovery. It has been proposed that the principal modes of the antibiotic actions result from an interaction of these antibiotics with the cell membrane of the target microorganisms. These antibiotics then adopt an antiparallel β-sheet conformation with amphiphilicty, which disrupts cell membrane.[1–4] In addition, so far, no resistance has been found for the antibiotics, because it requires significant alteration of the lipid composition of the cell membrane.[6] However, GS and TA have the high hemolytic activity, preventing their direct use in combating the microbial resistance.[1–3] In order to find drug candidates with high antimicrobial and low hemolytic activities, many analogues of GS and TA have been designed and synthesized.[1–3] For example, single substitution of Gln^6 of the natural TA with a cationic amino acid residue results in significant increase of a therapeutic index.[3c] On the other hand, a therapeutic index of GR and its analogues has not been studied. Recently, we measured the hemolytic activities against human erythrocytes of GR and [D-Ala$^{6,6'}$]-GR (**2**), which has D-Ala residues in place of D-Tyr$^{6,6'}$ residues. (Fig. 1) The hemolytic activity of GR

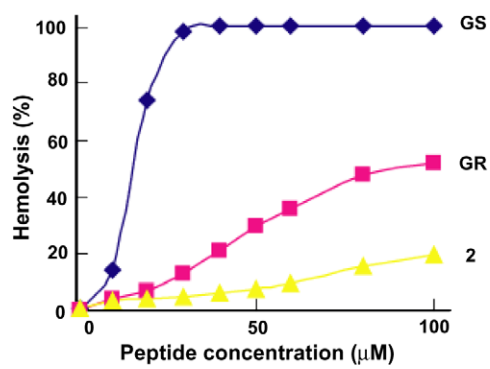

Figure 1. Dose dependence curves of hemolysis (%) induced by GS, GR and [D-Ala$^{6,6'}$]-GR (**2**).

was very low compared with that of GS. Further, the substitution of D-Tyr$^{6,6'}$ residues of GR with D-Ala residues results in decrease of the hemolytic activity compared with that of GR. The results indicated that the structural modifications at D-Tyr$^{6,6'}$ residues of GR are beneficial to identification of novel antibiotic candidates without hemolytic activity. Therefore, we planed to synthesize GR analogues that possess various D-amino acid residues in place of D-Tyr$^{6,6'}$ residues to find other candidates with both high antimicrobial and low hemolytic activities.

In the present account, we designed and synthesized novel fifteen GR analogues (**1–15**), which have various D-amino acid resi-

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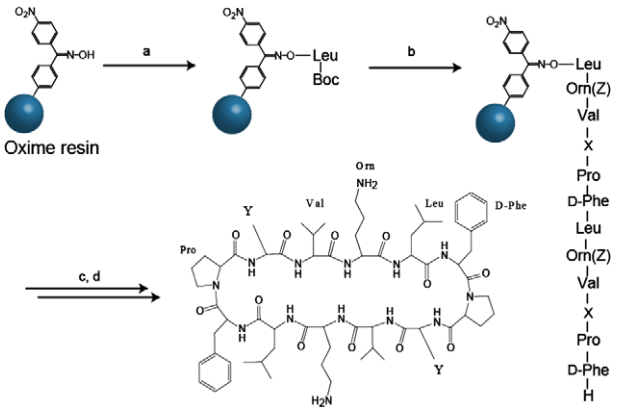

Scheme 1. Syntheses of GR and Its Analogues (**1–15**). Reagents and conditions: (a) Boc-Leu-OH (3 equiv), DCC (3 equiv) in DCM 24 h; (b) Boc-amino acid (3 equiv), BOP(3 equiv), HOBt (3 equiv), NEt_3 (6.5 equiv) in DMF 45 min Deprotection 25% TFA/DCM 30 min; (c) NEt_3 (2 equiv), AcOH (2 equiv) in 1,4-dioxane 24 h; (d) H_2 10% Pd/C, HCl (2 equiv) in MeOH and 1,4-dioxane, 2 days. (X = Gly, D-Ala, D-Nva, D-Val, D-Leu, D-Phe, D-Nle, D-Gln, D-Glu(OBzl), D-Thr(Bzl), D-Ser(Bzl), D-His(Bzl), D-Lys(*Z*), D-Orn(*Z*), and D-Arg(NO_2). Y = Gly, D-Ala, D-Nva, D-Val, D-Leu, D-Phe, D-Nle, D-Gln, D-Glu, D-Thr, D-Ser, D-His, D-Lys, D-Orn, and D-Arg).

dues (D-AA = Gly, D-Ala, D-Nva, D-Val, D-Leu, D-Phe, D-Nle, D-Gln, D-Glu, D-Thr, D-Ser, D-His, D-Lys, D-Orn, and D-Arg) in place of D-Tyr$^{6,6'}$ residues.

In the synthesis of gratisin and its analogues, a protected linear precursor oxime, H-(-D-Phe-Pro-X-Val-Orn(Z)-Leu)$_2$-oxime, (X = Gly, D-Ala, D-Nva, D-Val, D-Leu, D-Phe, D-D-Nle, D-Gln, D-Glu(OBzl), D-Thr(Bzl), D-Ser(Bzl), D-His(Bzl), D-Lys(Z), D-Orn(Z), and D-Arg(NO_2)), were prepared by using Boc-solid phase peptide synthesis on resin (Loading of oxime group: 0.6 mmol/g resins).[7] (Scheme 1) Leu residue as a C-terminal amino acid residue was used based on the propensity of the biosynthetic precursor of GS, TA and GR to form a conformation highly favorable for head-tail cyclization.[1,2,4] The formation of the cyclic peptide by the cyclization-cleavage of H-(-D-Phe-Pro-X-Val-Leu-Orn(Z)-Leu)$_2$-oxime on resin was performed in 1,4-dioxane with 2 equiv of triethylamine and acetic acid for 1 day at room temperature.[7] The cyclic product obtained was purified by means of sephadex LH-20 column chromatography, followed by recrystallization. The cyclizations gave cyclo[-Val-Leu-Orn(Z)-Leu-D-Phe-Pro-X-]$_2$, in 50–70% yields. The removal of all the masking groups by hydrogenolysis yielded the corresponding antibiotics **1–15**. The homogeneities of the antibiotics were confirmed by thin-layer chromatography, high performance liquid chromatography (HPLC), elemental analysis, and fast-atom bombardment (FAB) mass spectrometry.

First, GR peptides **1–7** having D-amino acids residues with hydrophobic aliphatic and aromatic side chains in place of D-Tyr$^{6,6'}$ residues were examined, in order to find novel antibiotic candidates with high antimicrobial and low hemolytic activities. The results are summarized in Table 1 and Figure 2. [Gly$^{6,6'}$]-GR (**1**) almost showed antibacterial activities against both Gram-positive and Gram-negative bacteria. On the other hand, GR peptides **2–7** having D-amino acids residues with hydrophobic aliphatic and aromatic side chains in 6, 6′ position showed the strong against Gram-positive bacteria, but almost showed the activity against Gram-negative bacteria. Its activity against Gram-positive bacteria increased according to increasing of the degree of effective hydrophobicity of their molecular, which was showed by the retention times of each antibiotic from ODS column.[8] (Table 1). The results indicated that the presence of D-amino acid residues with hydrophobic side chains is effective for the interaction with Gram-positive bacterial membrane, while not for Gram-negative bacterial membrane. However, the hemolytic values of GR peptides **1–7** also

Table 1
Antibiotic activity and HPLC retention times (RT) of GS, GR and GR peptides **1–7**

No.	Peptides	MIC[a] (μg/mL)						rt[b] (min)
		A	B	C	D	E	F	
	GS	3.13	3.13	3.13	3.13	25	25	95.0
	GR	6.25	6.25	12.5	6.25	100	100	22.0
1	[Gly$^{6,6'}$]-GR	100	100	>100	100	>100	>100	12.5
2	[D-Ala$^{6,6'}$]-GR	12.5	12.5	25	12.5	>100	>100	21.0
3	[D-Nva$^{6,6'}$]-GR	12.5	12.5	25	12.5	>100	>100	24.0
4	[D-Val$^{6,6'}$]-GR	6.25	6.25	12.5	12.5	>100	>100	31.0
5	[D-Leu$^{6,6'}$]-GR	6.25	6.25	12.5	12.5	100	>100	32.5
6	[D-Phe$^{6,6'}$]-GR	6.25	6.25	12.5	12.5	100	>100	34.5
7	[D-Nle$^{6,6'}$]-GR	6.25	6.25	6.25	3.13	50	100	37.0

[a] MIC (Minimum Inhibitory concentration) was determined by microplate dilution method with 10^6 organisms per ml. The microorganisms employed in the assays were *Bacillus subtilis* NBRC 3513 (A), *Bacillus megaterium* ATCC 19213 (B), *Staphylococcus epidemidis* NBRC 12933 (C), *Staphylococcus aureus* NBRC 12732 (D), *Pseudomonas aeruginosa* NBRC 3080 (E) and *Escherichia coli* NBRC 12734 (F).

[b] TSK-Gel C18 column (4.6 × 250 mm, 10 mm particle size, Tosoh Co., Japan) was used; flow rate, 1 ml/min; solvent, MeOH-0.1%TFAaq (65:35); monitoring wavelength, 220 nm.

increased according to increasing of the degree of effective hydrophobicity of their molecular. (Fig. 2) Thus, the increase of the hydrophobicity of GR peptide molecules is facilitating its binding with the membrane of Gram-positive bacteria, while detrimental to the interaction with mammalian cell membrane, which have limited its therapeutic value to topical applications. (Table 1 and Fig. 2).

Next, GR peptides **8–15** having D-amino acids residues with various functional hydrophilic side chains in place of D-Tyr$^{6,6'}$ residues were synthesized, because the introduction to GR of D-amino acid residues with hydrophilicity could expected to possess limited toxicity to erythrocytes.[2e,3c] The retention times of GR and GR peptides **8–15** from ODS column showed in Table 2. The results indicated that these GR peptides **8–15** have lower hydrophobicity of their molecular than that of GR. As expected all of GR analogues **8–15** exhibited substantially reduced hemolytic activities. (Fig. 3) Thus, a good correlation between the hemolytic activity and retention times of GR peptides **1–15** was found. (Fig. 4) [D-Gln$^{6,6'}$]-GR (**8**) with the neutral amide side chains showed 1/2 activity of GR against Gram-positive bacteria and no activity toward Gram-negative bacteria. On the other hand, [D-Ser$^{6,6'}$]-, [D-Thr$^{6,6'}$]- and [D-Glu$^{6,6'}$]- GR (**9–11**) with the neutral hydroxyl and acidic side chains almost showed the antibiotic activity against Gram-positive and Gram-negative bacteria. Thus, the presence of D-amino acid residues with the neutral hydroxyl and the acidic side chains is almost effective for the interaction with both bacterial membrane and mammalian cell membrane. [D-His$^{6,6'}$]-GR (**12**) with weak basic

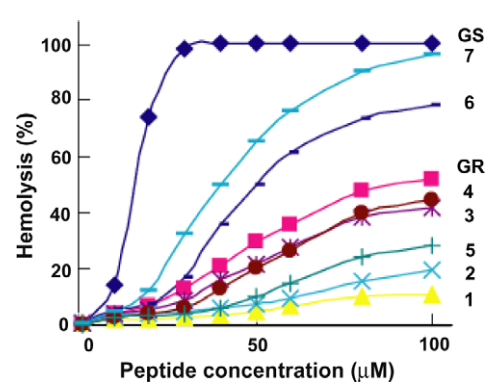

Figure 2. Dose dependence curves of hemolysis (%) induced by GS, GR and GR-peptides (**1–7**).

Table 2
Antibiotic activity and HPLC retention times (RT) of GR and GR peptides **8–15**.

No.	Peptides	MIC[a] (μg/mL)						rt[b] (min)
		A	B	C	D	E	F	
	GR	6.25	6.25	12.5	6.25	100	100	22.0
8	[D-Gln$^{6,6'}$]-GR	12.5	12.5	25	25	>100	>100	17.2
9	[D-Glu$^{6,6'}$]-GR	>100	>100	>100	>100	>100	>100	16.5
10	[D-Thr$^{6,6'}$]-GR	100	100	100	50	>100	>100	4.5
11	[D-Ser$^{6,6'}$]-GR	>100	>100	>100	100	>100	>100	3.5
12	[D-His$^{6,6'}$]-GR	12.5	12.5	25	12.5	>100	>100	2.2
13	[D-Lys$^{6,6'}$]-GR	6.25	6.25	6.25	6.25	12.5	25	2.2
14	[D-Orn$^{6,6'}$]-GR	6.25	6.25	6.25	6.25	25	25	2.0
15	[D-Arg$^{6,6'}$]-GR	6.25	3.13	6.25	3.13	12.5	12.5	2.0

[a] MIC (Minimum Inhibitory concentration) was determined by microplate dilution method with 10^6 organisms per ml. The microorganisms employed in the assays were *Bacillus subtilis* NBRC 3513 (A), *Bacillus megaterium* ATCC 19213 (B), *Staphylococcus epidemidis* NBRC 12933 (C), *Staphylococcus aureus* NBRC 12732 (D), *Pseudomonas aeruginosa* NBRC 3080 (E) and *Escherichia coli* NBRC 12734 (F).

[b] TSK-Gel C18 column (4.6 × 250 mm, 10 mm particle size, Tosoh Co., Japan) was used; flow rate, 1 ml/min; solvent, MeOH-0.1%TFAaq (65:35); monitoring wavelength, 220 nm.

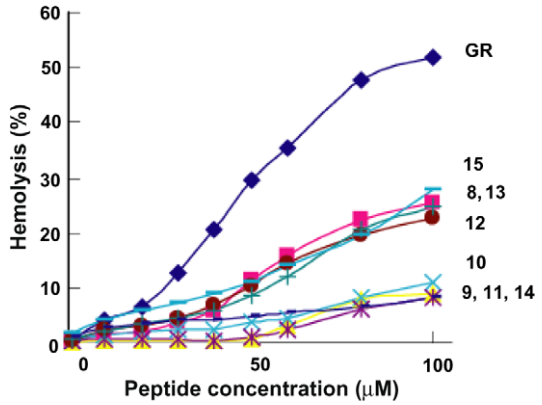

Figure 3. Dose dependence curves of hemolysis (%) induced by GR and GR-peptides (**8–15**).

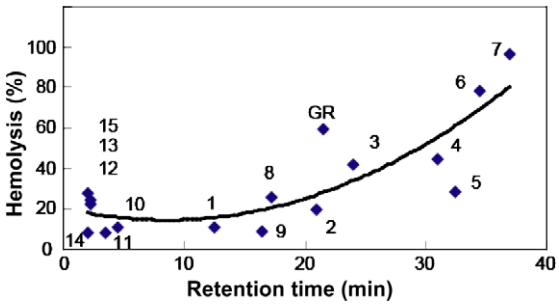

Figure 4. Correlation between hemolysis (%)[a] of GR peptides (**1–15**) and their retention time (min) in HPLC analysis.[b] [a]Percentage hemolysis of the peptides (100 μM) in 10% DMSO-buffer solution against human erythrocytes. [b]TSK-Gel C18 column (4.6 × 250 mm, 10 mm particle size, Tosoh Co., Japan) was used; flow rate, 1 ml/min; solvent, MeOH-0.1%TFAaq (65:35); monitoring wavelength, 220 nm.

imidazole side chains showed 1/2 activity of GR against Gram-positive bacteria and no activity toward Gram-negative bacteria. Significant further increase in the activity is achieved when the cationic amino acid residual D-Orn, D-Lys, and D-Arg is introduced into positions of D-Tyr $^{6,6'}$ residues. [D-Lys$^{6,6'}$]-,[9] and [D-Orn$^{6,6'}$]-GR (**13–14**) possessed the same activity as that of parent GR against Gram-positive bacteria. Further, the activity of [D-Arg$^{6,6'}$]-GR (**15**) is two times higher than that of parent GR against *Bacillus megaterium* ATCC 19213 and *Staphylococcus aureus* NBRC 12732. In addition, the antibiotics **13–15** showed the strong activity against Gram-negative bacteria. The antibiotic activities against *Pseudomonas aeruginosa* NBRC 3080 of **13**, and *Pseudomonas aeruginosa* NBRC 3080 and *Escherichia coli* NBRC 12734 of **15** are eight times higher than that of parent GR, respectively. It is interesting to note that the antibiotic potency against both Gram-positive and Gram-negative bacteria can increase and the hemolytic potency for mammalian cell membrane can decrease when cationic D-amino acid residues are incorporated into positions of D-Tyr$^{6,6'}$ of GR.

GR is an amphipathic peptide antibiotic with four hydrophobic side chains of Val$^{1,\ 1'}$ and Leu$^{3,\ 3'}$ residues on one side and two cationic side chains cons of Orn$^{2,\ 2'}$ residues on another side of the antiparallel β-sheet structure.[4e] GR with two cationic side chains is the strong antibacterial activity against Gram-positive bacteria, but is very weakly active against Gram-negative bacteria. On the other hand, polymixin B, which is an amphiliphic structure with polycationic nature, is strongly active against Gram-negative bacteria but is inactive or very weakly active against Gram-positive bacteria.[10] Polycationic antibiotics such as polymyxin B are considered to bind to the outer membrane of Gram-negative bacteria, leading to their disorganization and permeabillization.[11] In the present studies, we synthesized novel GR peptides **13–15** with the strong activity against both Gram-positive and Gram-negative bacteria, which have D-amino acid residues with cationic side chains in place of D-Tyr$^{6,6'}$ residues. In addition, we found that the GR peptides **13–15** have differential ionic interaction against the prokaryotic membrane and eukaryotic membrane. In other words, the dissociations of high antimicrobial and low hemolytic activities are caused by the additional positive charges of **13–15**.

In conclusion, we have found a novel position on the scaffold of GR at D-Tyr$^{6,6'}$ residues whose modification will significantly lower the unwanted hemolytic activity and simultaneously enhance the desired antibiotic activity. Our findings should be helpful in finding drug candidates with high antimicrobial and low hemolytic activities that are capable of combating microbial resistance. Currently, further synthetic studies of GR peptides with both strong antibiotic and low hemolytic activities are carrying on.

Supplementary data

Supplementary data (experimental procedures, and TLC, HPLC, MS and elemental analyses data for **1–15**) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.133.

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