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Novel gratisin derivatives with high antimicrobial activity and low hemolytic activity

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ABSTRACT

The substitution of each constituent amino acid residue of gratisin (GR) with Ala residue indicated that each side chain structure of the constituent amino acid residues affect largely the antibiotic and hemolytic activities of GR. Among them, the substitution of Pro residues at positions 5 and 5′ with a cationic amino acid residues (Lys and Arg) results the high antibiotic activity and the low toxicity against human blood cells. Thus, we have found a novel position on the scaffold of GR at Pro$^{5,5'}$ residues whose modification will significantly lower the unwanted hemolytic activity and enhance the desired antibiotic activity.

A remarkable increase of multidrug-resistant bacteria has become a serious threat to public health threat.[1] Among efforts to cope with this problem, the amphiphilic antibiotics such as gramicidin S (GS),[2,3] cyclo(-Val$^{1,1'}$-Orn$^{2,2'}$-Leu$^{3,3'}$-D-Phe$^{4,4'}$-Pro$^{5,5'}$-)$_2$, tyrocidine A (TA),[2,4] 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),[2b,5] cyclo(-Val$^{1,1'}$-Orn$^{2,2'}$-Leu$^{3,3'}$-D-Phe$^{4,4'}$-Pro$^{5,5'}$-D-Tyr$^{6,6'}$-)$_2$ are attractive targets for drug discovery. 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 amphiphilicity, which disrupts cell membrane.[2–5] 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 not only affect bacterial membranes but also mammalian cells such as erythrocytes. The high hemolytic activities of the peptides prevent their direct use in combating the microbial resistance.[2–4] The efforts to increase its therapeutic index, namely, minimizing its hemolytic activity while maintaining its high antibiotic activity, have been devoted.[2–4] 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.[4c] 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 GR analogues, which have various amino acid residues in place of D-Tyr$^{6,6'}$.[7] 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.

In the present studies, we performed a systematic approach to identify the structural determinants of its antibiotic and hemolytic properties that induced to dissociation of the two closely associated properties.

The syntheses of GR and its analogues were performed as shown in Scheme 1. The protected linear precursors were prepared by using Boc-solid phase peptide synthesis on oxime resin (loading of oxime group: 0.35 mmol/g resins).[8] 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 formations of the cyclic peptides by the cyclization-cleavage of the linear precursors on oxime resin were performed in 1,4-dioxane with 2 equiv of triethylamine and acetic acid for 1 day at room temperature.[7] All the masking groups were removed by thioanisole (50 equiv) and TFMSA (5 equiv) in TFA. The cyclic products obtained were purified by means of sephadex LH-20 column chromatography, followed by recrystallization. The purity and identity assessment of the products were confirmed by thin-layer chromatography, high performance liquid chromatography (HPLC), and fast-atom bombardment (FAB) mass spectrometry before determination of the biological activities.

First, GR peptides **1**–**6** having Ala residue in place of each constituent amino acid residue of GR were examined, in order to find

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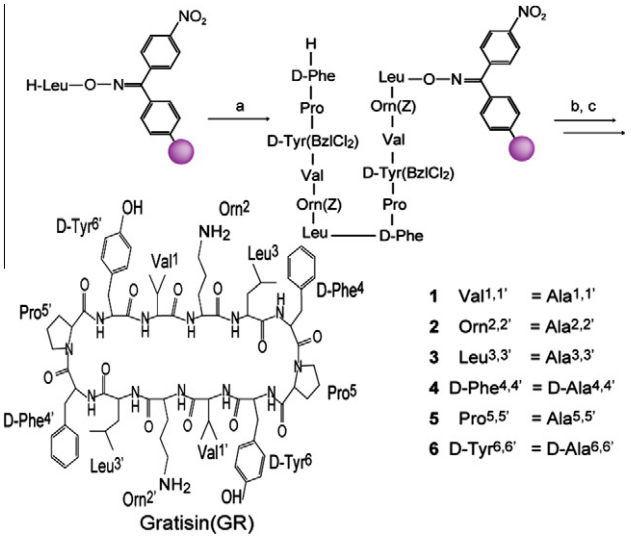

Scheme 1. Syntheses of GR and its Ala-substituted analogues **1**–**6**. Reagents and conditions; (a) Boc-amino acid (3 equiv), BOP (3 equiv), HOBt (3 equiv), NEt_3 (6.5 equiv) in DMF 90 min, deprotection 25% TFA/DCM 30 min; (b) NEt_3 (2 equiv), AcOH (2 equiv) in 1,4-dioxane, 24 h; (c) thioanisole (50 equiv) and TFMSA (5 equiv) in TFA, 2 days.

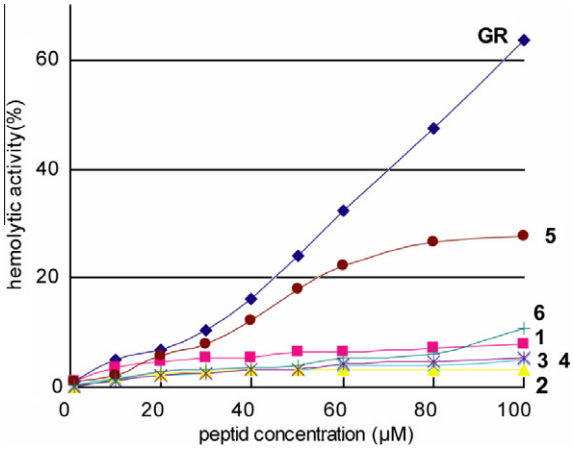

Figure 1. Dose dependence curves of hemolysis (%) induced by GR and its Ala-substituted analogues **1**–**6**.

novel antibiotic candidates with high antimicrobial and low hemolytic activities. The results are summarized in Table 1 and Fig. 1. [$Ala^{2,2'}$]-GR (**2**), [$Ala^{3,3'}$]-GR (**3**), and [D-$Ala^{4,4'}$]-GR (**4**) showed almost no antibacterial activities against both Gram-positive and Gram-negative bacteria. The hemolytic values of **2**, **3**, and **4** showed considerable decrease in compared with that of GR. The results indicated that the presences of $Orn^{2,2'}$, $Leu^{3,3'}$, and D-$Phe^{4,4'}$ residues in GR is necessary to exhibit the antibiotic activity and hemolytic activity, in other words, its modifications are unlikely to improve the antibacterial activity of GR. On the other hand, [$Ala^{1,1'}$]-GR (**1**), [$Ala^{5,5'}$]-GR (**5**), and [D-$Ala^{6,6'}$]-GR (**6**) showed 1/2 activity of GR against Gram-positive bacteria, but showed no antibiotic activity against Gram-negative bacteria. In addition, the hemolytic values of **1**, **5**, and **6** showed considerable decrease in compared with that of GR. In the previous studies, we have reported 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.[7] The results suggested that the modifications of $Val^{1,1'}$ and $Pro^{5,5'}$ residues are likely to improve the antibacterial and hemolytic activity of GR (Table 1 and Fig. 1).

To further exploit the potential of the sensitivity of the biological activities to the $Val^{1,1'}$ and $Pro^{5,5'}$ residues, GR analogues **7**–**10** with various amino acid residues in place of $Val^{1,1'}$ and $Pro^{5,5'}$ residues were prepared (Fig. 2). Syntheses of these GR analogues were accomplished with the method illustrated in Scheme 1. After the characterization of the cyclic peptide products, they were subjected to the same biological activity determination.

[$Lys^{1,1'}$]-GR (**7**) showed no antibiotic activities against both Gram-positive and Gram-negative organisms, and very low hemolytic activity against human blood cells (Table 2 and Fig. 3). The results indicated that the presence of amino acid residues with hydrophobic side chains in positions 1 and 1′ is important for exhibiting both antibiotic and hemolytic activities of GR. Next, the biological activities of GR peptides [$Ser^{5,5'}$]-GR (**8**), [$Lys^{5,5'}$]-GR (**9**), and [$Arg^{5,5'}$]-GR (**10**) having amino acid residues with the neutral hydroxyl and cationic side chains in place of $Pro^{5,5'}$ residues were compared with that of [$Ala^{5,5'}$]-GR (**5**) and GR (Table 2 and Fig. 3). [$Ser^{5,5'}$]-GR (**8**) with the neutral hydroxyl chains showed 1/2–1/8 activity of GR against Gram-positive bacteria, and no activity toward Gram-negative bacteria. In addition, **8** showed very low hemolytic activity against human blood cells. The biological activities of **8** are similar to that of Ala-substituted analogue (**5**). The results suggested that the presence of Pro residues at the 5,5′ position of GR is not necessary for exhibiting the antibiotic activity against Gram-positive bacteria, but important for exhibiting the activities against Gram-negative bacteria and human blood cell.

Significant further increase in the activity is achieved when the cationic amino acid residues are introduced into 5,5′ positions of GR, in comparison to the parent GR and Ala-substituted analogue (**5**) at same positions.

Table 1
Antibiotic activities of GR and its Ala-substituted analogues **1**–**6**[a]

	A	B	C	D	E	F
GR	6.25	6.25	12.5	6.25	100	100
1	12.5	12.5	25	6.25	>100	>100
2	>50	>50	>50	>50	>50	>50
3	100	100	>100	100	>100	>100
4	>100	>100	>100	>100	>100	>100
5	12.5	25	50	12.5	>100	>100
6	12.5	12.5	25	12.5	>100	>100

[a] MIC (minimum inhibitory concentration) was determined by a medium 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).

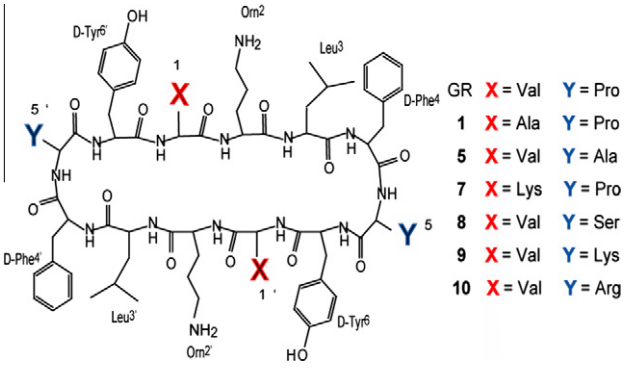

Figure 2. Primary structures of GR and its analogues (**1**, **5**, and **7**–**10**) with various amino acid residues at positions of $Val^{1,1'}$ and $Pro^{5,5'}$.

Table 2
Antibiotic activities of GR and its analogues (**1**, **5**, and **7**–**10**) with various amino acid residues at positions of Val[1,1′] and Pro[5,5′][a]

	A	B	C	D	E	F
GR	6.25	6.25	12.5	6.25	100	100
1	12.5	12.5	25	6.25	>100	>100
7	>100	>100	>100	>100	>100	>100
5	12.5	25	50	12.5	>100	>100
8	25	50	50	12.5	>100	>100
9	6.25	6.25	6.25	6.25	100	25
10	6.25	6.25	6.25	6.25	50	12.5

[a] MIC (minimum inhibitory concentration) was determined by a medium 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).

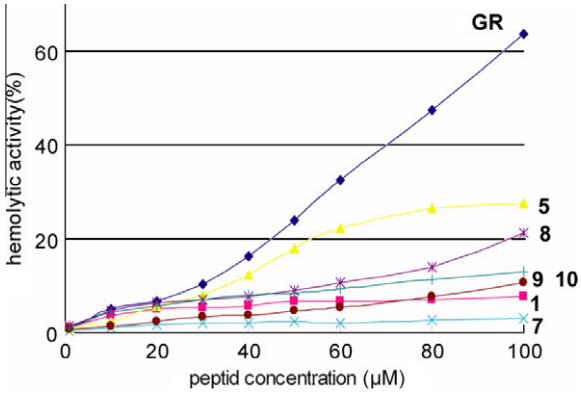

Figure 3. Dose dependence curves of hemolysis (%) induced by GR and its analogues (**1**, **5**, and **7**–**10**) with various amino acid residues at positions of Val[1,1′] and Pro[5,5′].

[Lys[5,5′]]-GR (**9**) and [Arg[5,5′]]-GR (**10**) with basic side chains showed similar activities against Gram-positive microorganisms and higher activities than that of parent GR against Gram-negative microorganisms. Among them, the antibiotic activities against *Pseudomonas aeruginosa* NBRC 3080 and *Escherichia coli* NBRC 12734 of **10** are two and eight times higher than that of parent GR, respectively. In addition, **9** and **10** displayed stronger inhibitory activity against *E. coli* NBRC 12734 compared to *P. aeruginosa* NBRC 3080. Further, the GR analogues **9** and **10** showed very low toxicity against human erythrocytes compared with that of GR. Thus, it is interesting to note that significant further increase in therapeutic index is achieved when Pro[5,5′] residues of GR were replaced by cationic amino acid residual Lys or Arg, in comparison to the Ala-substituted analogue at the same position and parent GR. Similar results were reported when cationic side chain was introduced into the positions of D-Tyr[6,6′] residues.[7] Recently, Qin et al. reported in studies of TA that the additional positive charge will greatly strengthen binding affinity of the resulting TA analogues for the prokaryotic membrane that contains predominantly negatively charged phospholipids, whereas its effect on the analogue's affinity for eukaryotic membrane will be much less significant because of the zwitterionic nature of the phospholipids in mammalian cells.[4c] Similar differential ionic interaction might also be responsible for the increase in therapeutic index when positively charged side chains are introduced into the position of Pro[5,5′] and D-Tyr[6,6′] of GR.

Next, CD spectra of **5**, **8**–**10** and GR were measured in methanol, in order to investigate the structure–activity relationship of **5** and **8**–**10** (Fig. 4). CD spectra of **5** and **8**–**10** were observed a curve similar to each other, but different from that of GR, indicating that the

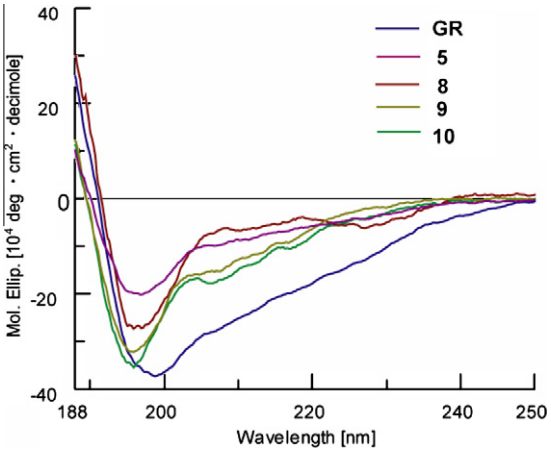

Figure 4. CD spectra of GR and its analogues (**5** and **8**–**10**) with various amino acid residues at positions of Pro[5,5′] in methanol.

conformation of these analogues in methanol are similar to each other, but different from that of GR. Thus, the presence of Pro[5,5′] residues with pyrrolidine ring is important for maintaining the GR-like conformation. These results suggested that the replacements of Pro [5,5′] residues may be partially effective through a structural change in its biological activity of **5** and **8**–**10**.

In the present studies, we synthesized novel GR peptides **9** and **10** with the strong activity against both Gram-positive and Gram-negative bacteria, which have amino acid residues with cationic side chains in place of Pro[5,5′] residues. In addition, we found that the GR peptides **9** and **10** 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 **9** and **10**. 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.

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Supplementary data

Supplementary data (experimental procedures, TLC, HPLC and MS data for **1**–**10**) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.10.122.

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