Hybrid Analogues of Gramicidin S and Gratisin

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Gramicidin S (GS), cyclo(-Val-Orn-Leu-D-Phe-Pro-)$_2$[1,2], and Gratisin (GR), cyclo(-Val-Orn-Leu-D-Phe-Pro-D-Tyr-)$_2$[3~9],[†] are potent cyclopeptide antibiotics and adopt an antiparallel β-sheet conformation[10~12] (Fig. 1). In the studies of the structure-activity relationship of these antibiotics, the mode of action has been proposed that when these antibiotics interact with a cell membrane of target microorganisms, they adopt antiparallel β-sheet conformations with C$_2$ symmetry and results in disruption of its cell membrane.[1~9,13] In addition, no resistance has been found for the antibiotics, because it requires significant alteration of the lipid composition of the cell membrane.[14] In view of the fact that widespread antibiotic resistance has become a serious threat to public health[15~18], these amphiphilic antibiotics are attractive targets for new drug discovery.

In these studies, we would like to report the design and synthesis of a new hybrid analogue, cyclo(-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-D-Tyr-) (**7a**), containing both the sequences of the (GS) pentapeptide (-Val-Orn-Leu-D-Phe-Pro-) and the (GR) hexapeptide (-Val-Orn-Leu-D-Phe-Pro-D-Tyr-) in the molecule (Fig. 1). The antiparallel β-sheet conformations of GS and GR place the charged Orn side chains on one side of the plane of the molecule and the hydrophobic Val and Leu side chains on the other side, and this plays an important role for exhibiting high antibiotic activity.[2] If each conformation of the partial sequences in the GS and GR molecules held in the hybrid analogue (**7a**), **7a** may possess the side-chain arrangement required for exhibiting the activity of GS and GR, even though **7a** is not C$_2$ symmetric. Further, we synthesized the two cycloundecapeptides, cyclo(-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-D-Tyr-Pro-) (**7b**) and cyclo(-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-Pro-D-Phe-D-Tyr-) (**7c**), which are hybrids of GS and two GR peptides possessing the high antibiotic activity, cyclo(-Val-Orn-Leu-D-Phe-D-Tyr-Pro-)$_2$ (**GR2**) and cyclo(-Val-Orn-Leu-Pro-D-Phe-D-Tyr-)$_2$ (**GR3**), respectively (Fig.1).

The synthesis of peptide **7a** was performed by a conventional liquid phase method. (Fig. 2) Boc-D-Tyr(BzlCl$_2$)-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl (**1a**)[††] and Boc-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl (**2a**) were synthesized from Pro benzyl ester by step-by-step elongation using 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDCI) and 1-hydroxybenzotriazole, and then saponified to give Boc-hexapeptide (**3a**) and Boc-pentapeptide (**4a**). Compound **3a** was converted into the corresponding succinimide ester (**3a′**) with EDCI and *N*-hydroxysuccinimide (HONSu).
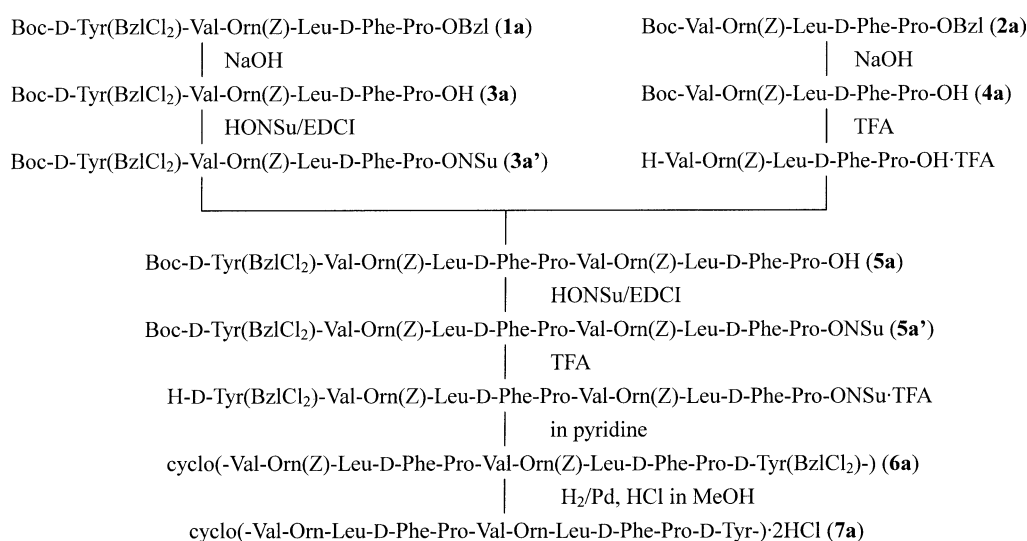
Fig. 1.   Primary structure of gramicidin S (**GS**), gratisin peptides (**GR**, **GR2**, and **GR3**) and its hybrid analogous (**7a**~**7c**).

	1	2	3	4	5	6	7	8	9	10	11	12
GS.	cyclo(-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-)											
GR.	cyclo(-Val-Orn-Leu-D-Phe-Pro-D-Tyr-Val-Orn-Leu-D-Phe-Pro-D-Tyr-)											
GR2.	cyclo(-Val-Orn-Leu-D-Phe-D-Tyr-Pro-Val-Orn-Leu-D-Phe-D-Tyr-Pro-)											
GR3.	cyclo(-Val-Orn-Leu-Pro-D-Phe-D-Tyr-Val-Orn-Leu-Pro-D-Phe-D-Tyr-)											

	1	2	3	4	5	6	7	8	9	10	11
7a.	cyclo(-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-D-Tyr-)										
7b.	cyclo(-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-D-Tyr-Pro-)										
7c.	cyclo(-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-Pro-D-Phe-D-Tyr-)										

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† Amino acid residues with no prefix are of L-configuration. The abbreviations of amino acids and peptides are in accordance with the rules of IUPAC-IBU commission of Biological Nomenclature.

†† Abbreviations used are as follows: Boc, *t*-butoxycarbonyl; Z, benzyloxycarbonyl; OBzl, Benzyloxy; BzlCl$_2$, 2,6-dichlorobenzyl.

Fig. 2.   Synthetic scheme of cyclo(-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-D-Tyr-) (**7a**).

Boc-D-Tyr(BzlCl₂)-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl (**1a**)   Boc-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl (**2a**)
   | NaOH | NaOH

Boc-D-Tyr(BzlCl₂)-Val-Orn(Z)-Leu-D-Phe-Pro-OH (**3a**)   Boc-Val-Orn(Z)-Leu-D-Phe-Pro-OH (**4a**)
   | HONSu/EDCI | TFA

Boc-D-Tyr(BzlCl₂)-Val-Orn(Z)-Leu-D-Phe-Pro-ONSu (**3a'**)   H-Val-Orn(Z)-Leu-D-Phe-Pro-OH·TFA

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Boc-D-Tyr(BzlCl₂)-Val-Orn(Z)-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-OH (**5a**)
   | HONSu/EDCI

Boc-D-Tyr(BzlCl₂)-Val-Orn(Z)-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-ONSu (**5a'**)
   | TFA

H-D-Tyr(BzlCl₂)-Val-Orn(Z)-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-ONSu·TFA
   | in pyridine

cyclo(-Val-Orn(Z)-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-D-Tyr(BzlCl₂)-) (**6a**)
   | H₂/Pd, HCl in MeOH

cyclo(-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-D-Tyr-)·2HCl (**7a**)

Table 1.   Yields and analytical data of intermediary products of hybrid analogs **7a∼7c**.

	yield (%)	mp (°C)	$[\alpha]_D^{25}$ (°) (c 0.5, DMF)	Formula(MW) [a]
2c. Boc-D-Phe-D-Tyr(BzlCl₂)-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl	51	215-216	-13.5	$C_{75}H_{91}O_{13}N_8Cl_2 \cdot 0.5H_2O$ (1392.5)
4c. Boc-D-Phe-D-Tyr(BzlCl₂)-Val-Orn(Z)-Leu-D-Phe-Pro-OH	90	198-200	-12.6	$C_{68}H_{85}O_{13}N_8Cl_2 \cdot 0.5H_2O$ (1302.4)
5a. Boc-D-Tyr(BzlCl₂)-Val-Orn(Z)-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-OH	55	190-194	-37.3 (c 1)	$C_{97}H_{128}O_{20}N_{13}Cl_2 \cdot 2.5H_2O(1896.1)$
5b. Boc-Val-Orn(Z)-Leu-D-Phe-D-Tyr(BzlCl₂)-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-OH	94	148-149	-37.8 (c 1)	$C_{97}H_{128}O_{20}N_{13}Cl_2 \cdot 1.5H_2O(1878.1)$
5c. Boc-Val-Orn(Z)-Leu-Pro-D-Phe-D-Tyr(BzlCl₂)-Val-Orn(Z)-Leu-D-Phe-Pro-OH	95	162	-16.5	$C_{97}H_{128}O_{20}N_{13}Cl_2 \cdot 2H_2O$ (1887.1)
6a. cyclo[-D-Tyr(BzlCl₂)-Val-Orn(Z)-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-]	35	237-240	-110.3	$C_{92}H_{118}O_{16}N_{13}Cl_2 \cdot 2.5H_2O(1778.0)$
6b. cyclo[-Val-Orn(Z)-Leu-D-Phe-D-Tyr(BzlCl₂)-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-]	45	231-233	-61.2	$C_{92}H_{118}O_{16}N_{13}Cl_2 \cdot 4H_2O$ (1805.0)
6c. cyclo[-Val-Orn(Z)-Leu-Pro-D-Phe-D-Tyr(BzlCl₂)-Val-Orn(Z)-Leu-D-Phe-Pro-]	37	220 (decomp.)	-42.5 (c 0.3)	$C_{92}H_{118}O_{16}N_{13}Cl_2 \cdot 2H_2O$ (1769.0)

a) The results of elemental analysis agreed with calculated values within ±0.3%.

b) Boc-D-Tyr(BzlCl₂)-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl (**1a**), . Boc-D-Tyr(BzlCl₂)-Val-Orn(Z)-Leu-D-Phe-Pro-OH (**3a**),
   Boc-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl (**2a** and **2b**),   Boc-Val-Orn(Z)-Leu-D-Phe-Pro-OH (**4a** and **4b**),
   Boc-Val-Orn(Z)-Leu-D-Phe-D-Tyr(BzlCl₂)-Pro-OBzl (**1b**), and Boc-Val-Orn(Z)-Leu-D-Phe-D-Tyr(BzlCl₂)-Pro-OH (**3b**),
   have been reported in the literature.[7,20]

Table 2. Yields and analytical data of hybrid analogs 7a~7c.

7a.	cyclo(-Val-Orn-Leu-D-Phe-Pro-D-Tyr-Val-Orn-Leu-D-Phe-Pro-)·2HCl
	yield, 65% ; mp 248-250 °C (decomp.) ; $[\alpha]_D^{25}$ -172.0 ° (c 0.5, EtOH).
	MS (FAB), m/z 1304 ($C_{69}H_{102}O_{12}N_{13}$; M+H$^+$).
	Amino acid analysis : Val, 2.20 ; Orn, 2.10 ; Leu, 2.20 ; Phe, 2.10 ; Pro, 1.92 ; Tyr, 1.00
	Found: C, 55.49 ; H, 7.58 ; N, 12.30%. Calcd for $C_{69}H_{102}O_{12}N_{13}$·2HCl·6.5H$_2$O:
	C, 55.41 ; H, 7.88 ; N, 12.17%.
7b.	cyclo(-Val-Orn-Leu-D-Phe-D-Tyr-Pro-Val-Orn-Leu-D-Phe-Pro-)·2HCl
	yield, 90% ; mp 228-231 °C (decomp.) ; $[\alpha]_D^{25}$ -164.2 ° (c 0.5, EtOH).
	MS (FAB), m/z 1304 ($C_{69}H_{102}O_{12}N_{13}$; M+H$^+$).
	Amino acid analysis : Val, 1.96 ; Orn, 1.90 ; Leu, 2.08 ; Phe, 2.04 ; Pro, 2.06 ; Tyr, 1.00
	Found: C, 55.88 ; H, 7.57 ; N, 12.23%. Calcd for $C_{69}H_{102}O_{12}N_{13}$·2HCl·6H$_2$O:
	C, 55.75 ; H, 7.86 ; N, 12.25%.
7c.	cyclo(-Val-Orn-Leu-Pro-D-Phe-D-Tyr-Val-Orn-Leu-D-Phe-Pro-)·2HCl
	yield, 99% ; mp 225 °C (decomp.) ; $[\alpha]_D^{25}$ -131.0 ° (c 0.5, EtOH).
	MS (FAB), m/z 1304 ($C_{69}H_{102}O_{12}N_{13}$; M+H$^+$).
	Amino acid analysis : Val, 2.10 ; Orn, 2.00 ; Leu, 2.20 ; Phe, 2.10 ; Pro, 1.90 ; Tyr, 1.00
	Found: C, 57.72 ; H, 7.87 ; N, 12.63%. Calcd for $C_{69}H_{102}O_{12}N_{13}$·2HCl·3.5H$_2$O:
	C, 57.45 ; H, 7.82 ; N, 12.62%.

Table 3. Antibiotic activities of **GS** and hybrid peptides 7a~7c.

Test organisms	Minimum inhibitory concentration (μg/ml) [a]			
	GS	**7a**	**7b**	**7c**
S. aureus Smith	3.13	3.13	3.13	6.25
S. aureus MS353 AO	3.13	3.13	3.13	6.25
S. aureus 0175	3.13	6.25	3.13	6.25
S. epidermidis ATCC 27626	3.13	3.13	3.13	6.25
S. pyogenes N.Y.5	3.13	3.13	3.13	3.13
S. pyogenes S-23	3.13	3.13	6.25	3.13
S. agalactiae ATCC 12386	3.13	3.13	3.13	6.25
M. luteus ATCC 9341	3.13	6.25	12.5	6.25
B. subtilis ATCC 6633	3.13	3.13	3.13	3.13
E. coli NIHJ-JC2	>100	>100	>100	>100
K. pneumoniae NCTC 9632	12.5	25	>100	>100
P. aeruginosa PA01	>100	>100	>100	>100

a) Minimum inhibitory concentration (μg/ml) was determined by an agar dilution method with 10^6 organisms per milliliter.

Coupling of **3a′** with pentapeptide derived from **4a** afforded the undecapeptide (**5a**), which was converted into the undecapeptide succinimide ester (**5a′**). Boc-group of **5a′** was removed by the action of trifluoroacetic acid (TFA), and then the succinimide ester was cyclized under high dilution (peptide concentration: 3×10^{-3} M) in pyridine at 45°C for 3 hours. The resulting product was purified by means of high-performance liquid chromatography, followed by recrystallization. The cycloundecapeptide (**6a**) was obtained in 35% yield. The removal of all the masking groups of **6a** by hydrogenolysis yielded **7a**. Peptides **7b** and **7c** were synthesized in a similar manner, in 45 and 37% cyclization yields, respectively. The homogeneity of hybrid analogs **7a~7c** was confirmed by means of thin-layer chromatography, amino acid analysis, HPLC, elemental analysis, and FAB mass spectrometry (Table 1 and 2).

The antibiotic activity of these hybrid analogues **7a~7c** and GS is summarized in Table 3. The hybrid analogue **7a** showed antibiotic activity against all Gram-positive microorganisms tested, and its activity is the same activity as that of GS. Other hybrid analogues **7b** and **7c** also showed strong activity. These results suggest that a secondary structure having the side-chain arrangement required for the antibiotic activity is present in these hybrid analogues, despite the fact that their primary structure is not C_2 symmetric.

To investigate the structure-activity relationship of these hybrid analogues, CD spectra of GS, GR peptides and the three hybrid analogues, **7a~7c**, were measured in aqueous solutions (Fig. 3). The shapes of the CD spectra of **7a~7c** are similar to the graphical average of the CD spectra of the GS and GR peptides, but the trough of the CD curves is slightly shallower than their graphical averages. The difference found in the ellipticities of these compounds seems to reflect the difference of the stability of their structures in aqueous solutions. We reported that the CD spectra of the GS and GR peptides reflect the ring features near the Pro residue, but not always the entire structure of the molecule.[7,19] The existence of additivity in CD spectra suggests that these hybrid analogs have both conformations around the β-turn part of the GS and GR peptides, in other words, structures with the side-chain arrangement required for exhibiting the activity of GS and GR peptides.

Further detailed conformational analysis of these hybrid peptides are needed in order to clearly understand the structure-activity relationship.
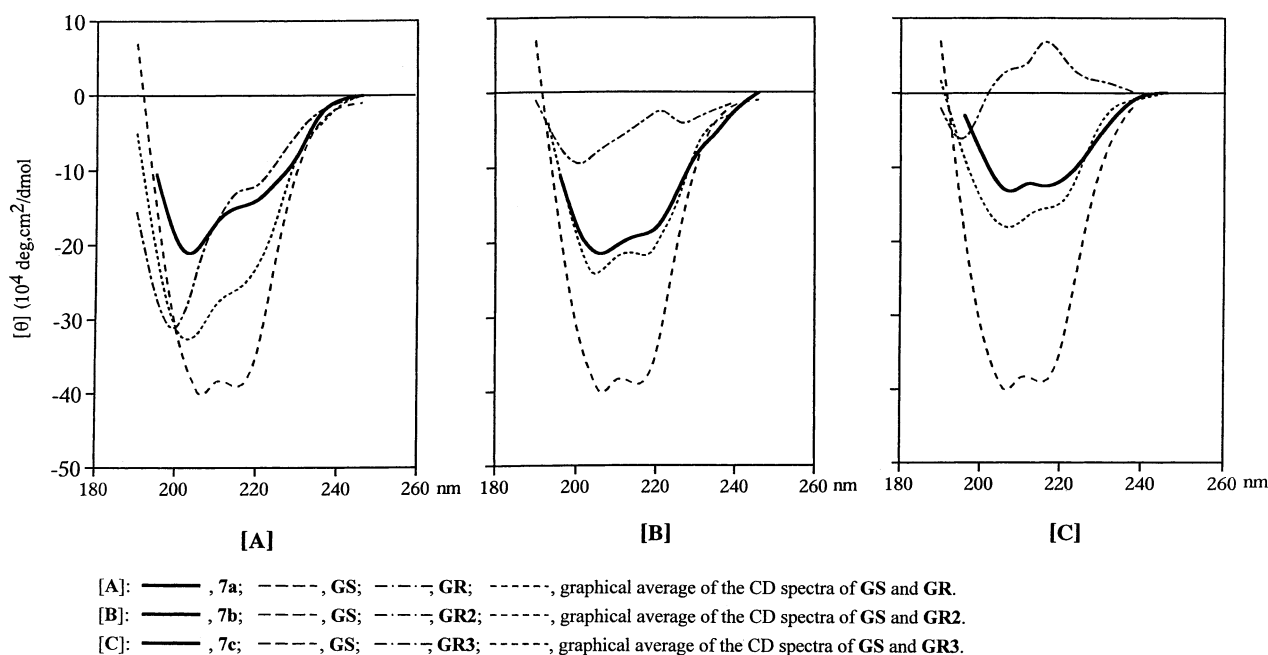
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Fig. 3.   CD spectra of **GS**, **GR** peptides and its hybrid analogs **7a~7c** in aqueous solution.

[A]: ——— , 7a; –––– , GS; –·–·– , GR; ----- , graphical average of the CD spectra of **GS** and **GR**.
[B]: ——— , 7b; –––– , GS; –·–·– , GR2; ----- , graphical average of the CD spectra of **GS** and **GR2**.
[C]: ——— , 7c; –––– , GS; –·–·– , GR3; ----- , graphical average of the CD spectra of **GS** and **GR3**.

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