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Development and Antifungal Efficacy of Hydrocarbon-Stapled Peptide Analogs Based on Human Antimicrobial Peptides for Candidiasis Therapy

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Abstract

KR-12, derived from the human antimicrobial peptide LL-37, maintains the antimicrobial properties of LL-37 while minimizing human cell toxicity. The KR-12 analog, named KR-12-pa, is characterized by its amphipathic α-helical structure, contributing to its strong antimicrobial and anti-inflammatory effects. In this study, we designed hydrocarbon-stapled peptide analogs based on KR-12-pa incorporating chiral non-natural amino acids at positions i and i+4. All stapled peptides were synthesized by solid-phase peptide synthesis method, verified by LC-MS. The stapled peptides showed broad-spectrum antifungal activities, with minimum inhibitory concentrations (MIC) ranging from 2 to 32 μg/mL, against pathogenic *Candida* strains including the fluconazole-resistant *Candida krusei* strain. Notably, a subset of these analogs, specifically S3, S5, and S7, demonstrated low human cell toxicity comparable to the original KR-12 peptide. Among these, S3 stood out for its balance of potent antifungal activity and minimal toxicity, making it a promising candidate for further development.

Further structural analysis through circular dichroism indicated that S3 maintains an α-helical conformation in buffer solutions, differing from the wild-type peptide. Preliminary *in vivo* assessments using the *Galleria mellonella* model underscored S3's reduced toxicity and heightened antifungal efficacy. Altogether, these results position the S3 stapled analog of KR-12-pa as a viable and effective option for treating candidiasis, highlighting its potential as a therapeutic innovation.

Introduction

Antimicrobial peptides (AMPs) are an important component of the innate immune system and are produced by almost all living organisms, from plants and insects to mammals, including humans. The ability to physically destroy bacterial cell membranes and induce lysis allows AMPs to target bacteria, fungi, and enveloped viruses and renders them a potential alternative to conventional antibiotics LL-37 is a natural antimicrobial peptide expressed in the human body.

❖ KR-12-pa

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LL-37      LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
KR-12                  KRIVQRIKDFLR
KR-12-pa               KRIVKRIKKFLR
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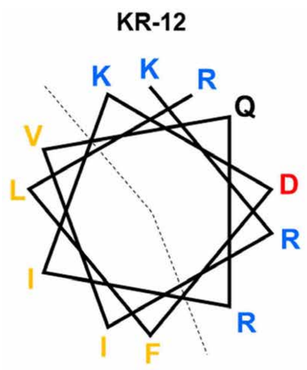
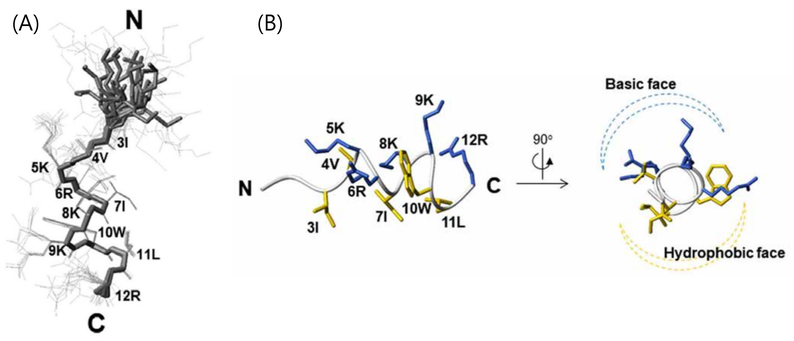


- Primary sequences and helical wheel diagrams of LL-37, KR-12, and KR-12-pa. Positively charged residues are in blue
- Negatively charged residues are in red. Hydrophobic residues are in yellow

❖ NMR structure of KR-12-pa



(A) Stereo view of twenty converged line structures of KR-12-pa for backbone heavy atoms
(B) The lowest target function structure of KR-12-pa

Results

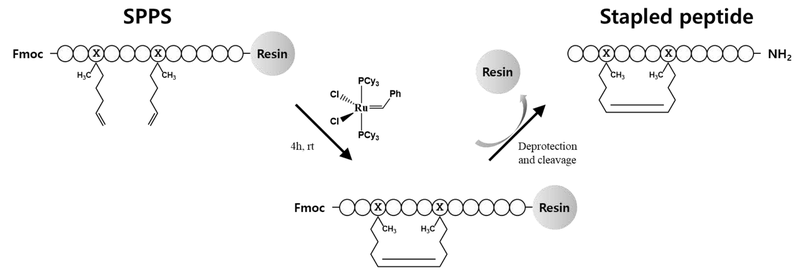
Design and synthesis of stapled peptide analogs based on KR-12-pa

Design of stapled peptides

		Sequence	MW Linear	MW Stapled
No stapled	WT	KRIVKRIKKWLR-NH_2	1623.10	-
Single stapled (i, i+4)	S1	**X**RIV**X**RIKKWLR-NH_2	1645.14	1617.08
	S2	K**X**IVK**X**IKKWLR-NH_2	1589.11	1561.01
	S3	KR**X**VKR**X**KKWLR-NH_2	1675.17	1647.11
	S4	KRI**X**KRI**X**KWLR-NH_2	1674.18	1646.12
	S5	KRIV**X**RIK**X**WLR-NH_2	1645.14	1617.08
	S6	KRIVK**X**IKK**X**LR-NH_2	1559.08	1531.03
	S7	KRIVKR**X**KKW**X**R-NH_2	1675.17	1647.11
	S8	KRIVKRI**X**KWL**X**-NH_2	1617.12	1589.07

- X : (S)-2-(4-pentenyl)Ala-OH

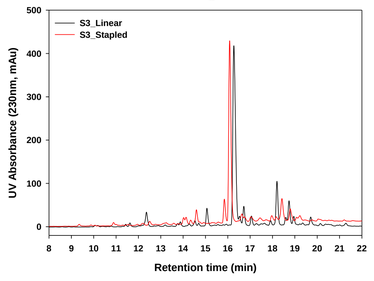
Synthetic scheme of stapled peptides



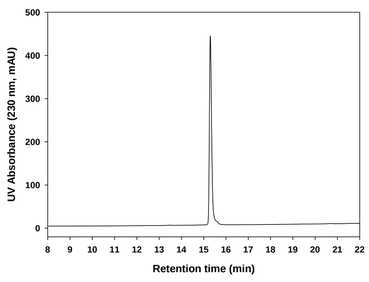
Preparation of KR-12-S3 stapled peptides

Instrumental conditions of peptide with LC-MS	
LC-MS system	Shimadzu-10AD/AB Sciex API2000
Column	Sunfire C18 (4.5 x 250 mm)
Buffer	A: 100% H_2O containing 0.05% TFA B: 100% ACN containing 0.05% TFA
Method	5-65% for 30 min
Detector	230 nm
Flow rate	1 mL/min

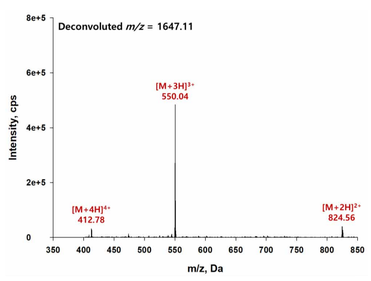
Cleavage



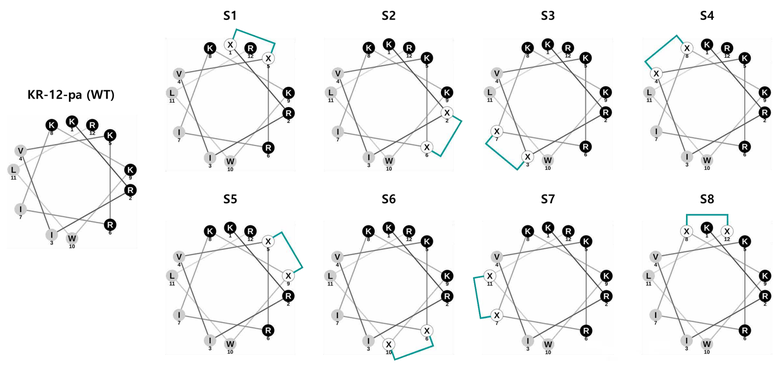
HPLC



LC-MS

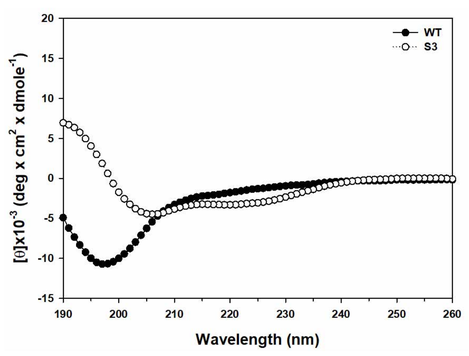


Helical wheel diagrams



Single stapled (i, i+4), Polar amino acids, Nonpolar amino acids

CD spectra of WT and S3



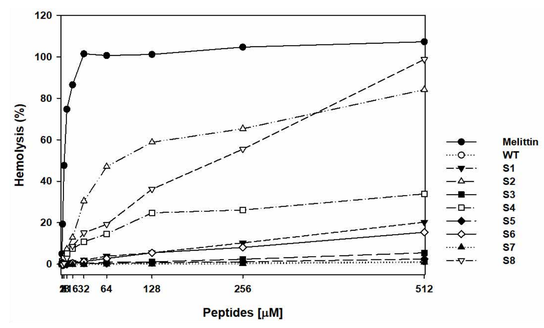
- Jasco, J-810
- 20 ℃
- 200 μM in 50 mM sodium phosphate

Antimicrobial activity

Strain		MIC (μg/mL) WT	S1	S2	S3	S4	S5	S6	S7	S8
C. parapsilosis	ATCC 22019	16	16	4	4	2	16	4	4	2
C. albicans	ATCC MYA-2876	64	16	4	8	4	32	16	16	4
C. glabrata	ATCC 90030	8	4	2	2	2	4	2	2	2
C. glabrata	ATCC 2001	>64	64	4	8	8	>64	8	16	8
C. krusei	ATCC 6258	64	16	4	4	4	16	8	16	4
C. tropicalis	ATCC 750	4	8	2	2	2	4	2	4	2

- Antifungal susceptibility strains : ATCC 22019, MYA-2876, 750
- Antifungal susceptible-dose dependent(SDD) strains : ATCC 90030, 2001
- Antifungal resistant strains : ATCC 6258

Hemolytic activity

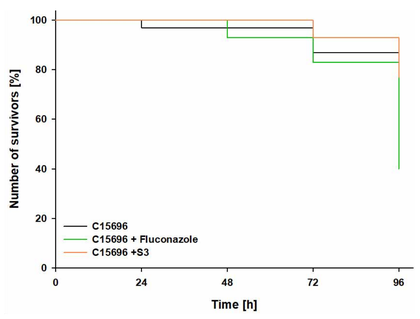


S5, S7 < WT < S3 < S6 < S1 < S4 < S2 < S8

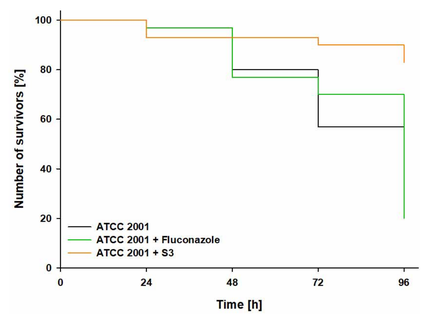
Effect of S3 stapled peptide from *Galleria mellonella*

Galleria mellonella larvae

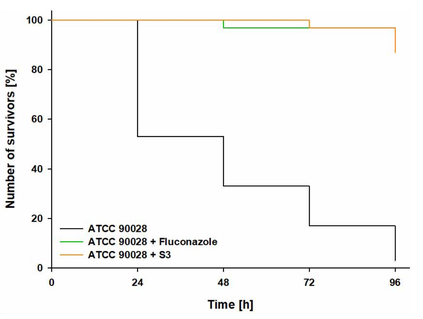
C. glabrata (C15696)



C. glabrata (ATCC 2001)



C. albicans (ATCC 90028)



Conclusion

Eight stapled peptides based on KR-12-pa, derived from the human antimicrobial peptide LL-37, were synthesized using solid-phase synthesis and purified by RP-HPLC. Among the eight stapled peptides, S3 demonstrated high antifungal activity and low hemolytic activity. The CD spectrum showed that S3 maintains an α-helical conformation in buffer solutions. Preliminary *in vivo* assessments of the S3 peptide, confirmed using *Galleria mellonella* larvae, showed low toxicity and heightened antifungal efficacy.

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