Toxin-Activating Stapled Peptides Discovered by Structural Analysis were Identified as New Therapeutic Candidates that Trigger Antibacterial Activity against *Mycobacterium Tuberculosis*

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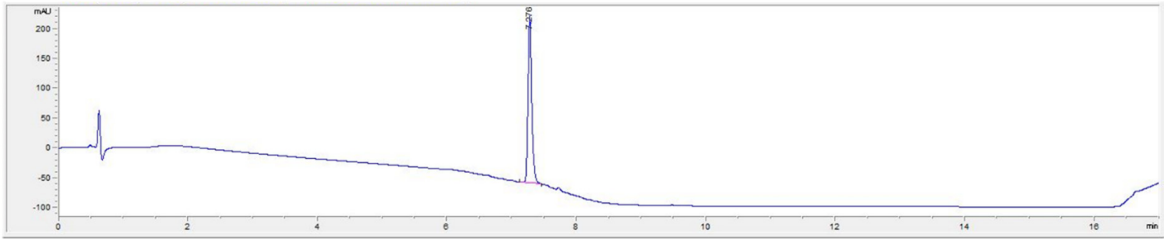
Contents

V26-Pep-8 (>95% purity)



FITC-V26-Pep-8 (>95% purity)

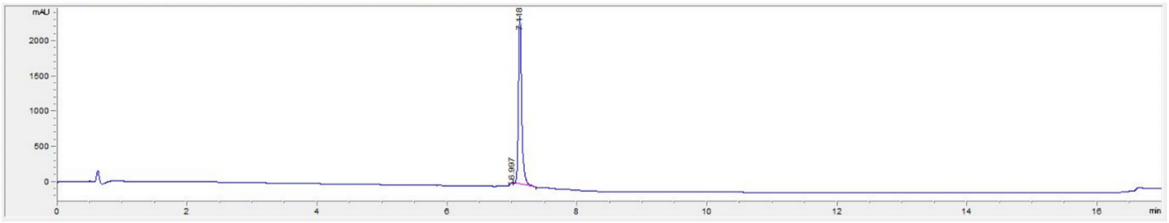


Figure S1: HPLC chromatogram of the purified peptides.

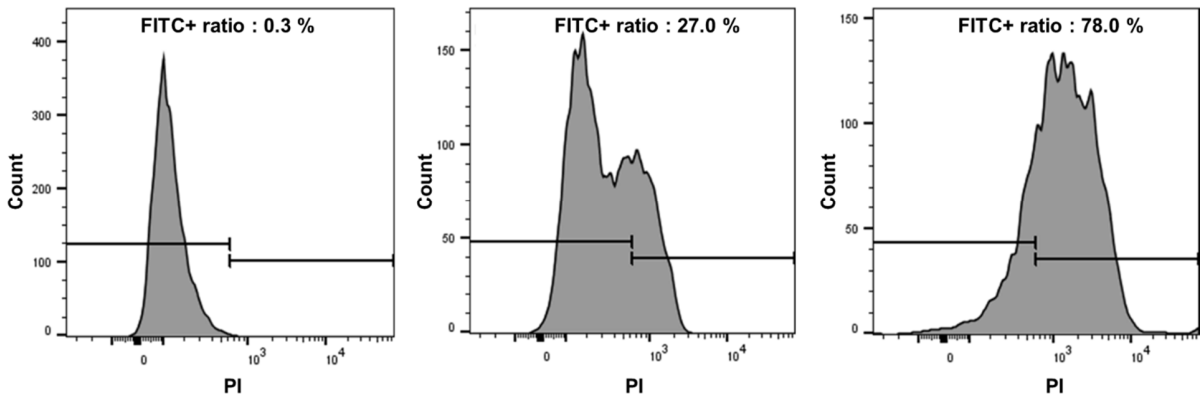
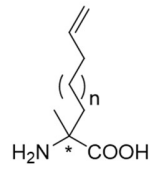


Figure S2: Flow cytometry data of *M. smegmatis* cells obtained from the untreated (negative control, left) group and the FITC-labeled peptide-added groups (linear and stapled forms in the middle and right, respectively).

a

Entry	Peptide	Sequence
7	**V26-SP-7**	DAELAVLR**R$_5$**LA**S$_5$**
8	**V26-SP-8**	**R$_8$**AELAVL**S$_5$**ELA
9	**V26-SP-9**	DAELAVL**S$_5$**ELA**S$_5$**

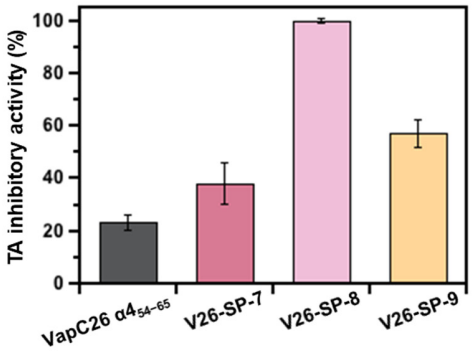


R$_5$: n = 1, * = *(R)*

R$_8$: n = 4, * = *(R)*

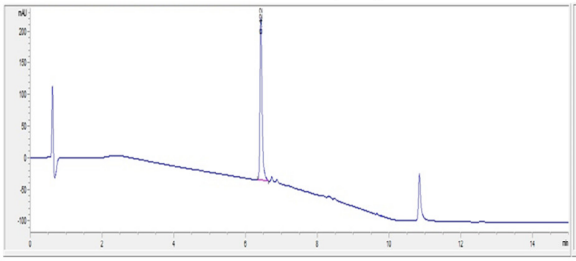
S$_5$: n = 1, * = *(S)*

b

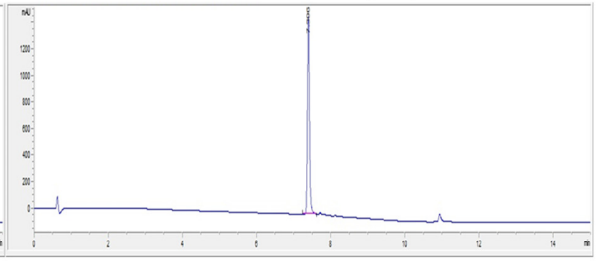


c

V26-SP-7 (>95% purity)



V26-SP-9 (>95% purity)



Peptide	Sequence	Chemical Formula	Exact Mass (M+ H$^+$)	Exact Mass (M+ Na$^+$)	Observed Mass
V26-SP-7	Ac-Asp-Ala-Glu-Leu-Ala-Val-Leu-Arg-R$_5$-Leu-Ala-S$_5$-NH$_2$	$C_{63}H_{108}N_{16}O_{17}$	1361.8151	1383.7971	1361.8169
V26-SP-9	Ac-Asp-Ala-Glu-Leu-Ala-Val-Leu-S$_5$-Glu-Leu-Ala-S$_5$-NH$_2$	$C_{62}H_{103}N_{13}O_{19}$	1334.7566	1356.7385	1334.7601

Figure S3: Data of the stapled peptides. **(a)** Stapled peptides synthesized based on VapC26 α4$_{54-65}$. **(b)** *In vitro* ribonuclease activity assay results of stapled peptides. The activity was normalized to that of **V26-SP-8**. The concentration of each peptide was 10 μM. Data are presented as the mean ± SD of three independent replicates. **(c)** HPLC chromatogram and HRMS analysis of two stapled peptides.

Supplementary Table S1: HRMS analysis of peptides.

Peptide	Sequence	Chemical Formula	Exact Mass ($M+H^+$)	Exact Mass ($M+Na^+$)	Observed Mass
V26-SP-8	Ac-R_8-Ala-Glu-Leu-Ala-Val-Leu-S_5-Glu-Leu-Ala-NH_2	$C_{61}H_{104}N_{12}O_{16}$	1261.7766	1283.7586	1283.7585
FITC-V26-SP-8	FITC-Ahx-R_8-Ala-Glu-Leu-Ala-Val-Leu-S_5-Glu-Leu-Ala-NH_2	$C_{86}H_{124}N_{14}O_{21}S$	1721.8859	1743.8678	1743.8689

Supplementary Table S2: Primers used for cloning.

pYUBDuet Vector-Rv0581-Rv0582		
1st PCR	Rv0581-BHI_F	accacagccaggatcc g atggacaagacgacggtc
	Rv0581-HindIII_r	atgcggccgcaagctt tcaccgctcaccgaagcc
2nd PCR	Rv0582-NdeI_F	agaaggagatatacat atgatcatcgacacgagt
	Rv0582-EcoRV-r	gtggccggccgatatc gc cggaatgacggtgaagcg