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Supplementary Information

Exploring the molecular structure of lipids in the design of artificial lipidated antifungal proteins

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1. Supplementary information

1-1. Amino acid sequence of LysM-Q (pI/Mw: 6.99 / 8739.65)

MCTTYTIKSGDTCYAISQARGISLSDFESWNAGIDCNNLQIGQVVCVSKPSTSTTPSPT
PSSSSNGFYPLQMRGGHHHHHH

1-2. Amino acid sequence of LysM-muGFP-Q (pI/Mw: 6.04 / 35078.23)

MCTTYTIKSGDTCYAISQARGISLSDFESWNAGIDCNNLQIGQVVCVSKPSTSTTPSPT
PSSSSNGHHHHHHSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFI
CTTGKLPVPWPTLVTTLTYGVLCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGTY
KTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVYITADKQKNGIKAYF
KIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSVLSKDPNEKRDHMVLLEDV
TAAGITHGMDELYRGGGGSLLQG

Brown: LysM2 domain
Blue: Linker sequences derived from PrChiA
Green: Hexahistidine tag
Purple: Q-tag (FYPLQMRGG)
Dark Green: muGFP
Orange: Q-tag (LLQG)

1-3. Molecular structure of lipid-modified peptides

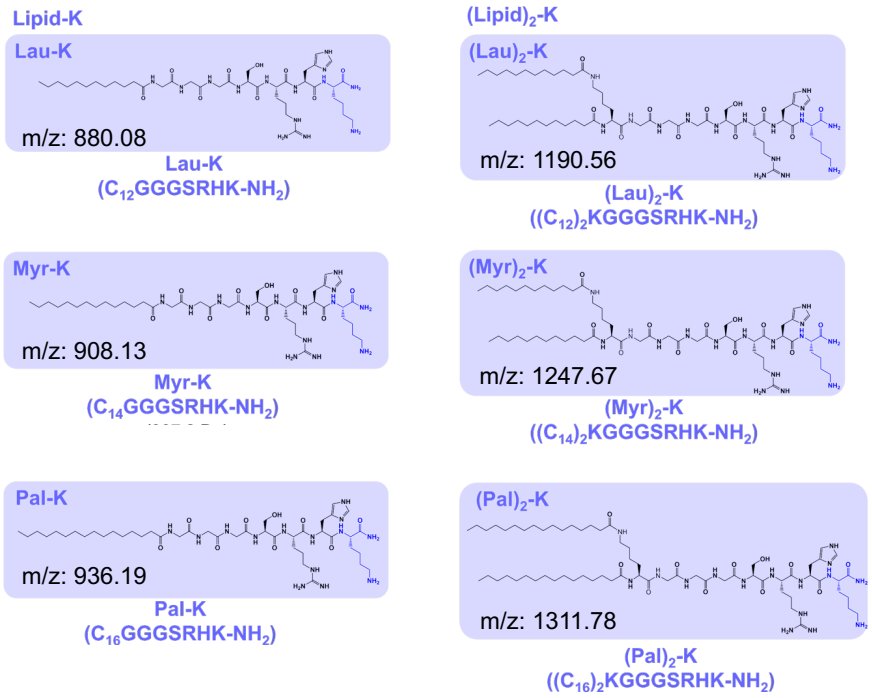


Figure S1. Chemical structures of lipid-G_3S-RHK and (lipid)$_2$-KG_3S-RHK with the values of theoretical molecular weight.

2. Supplementary results

2-1. Results of Fmoc solid phase peptide synthesis

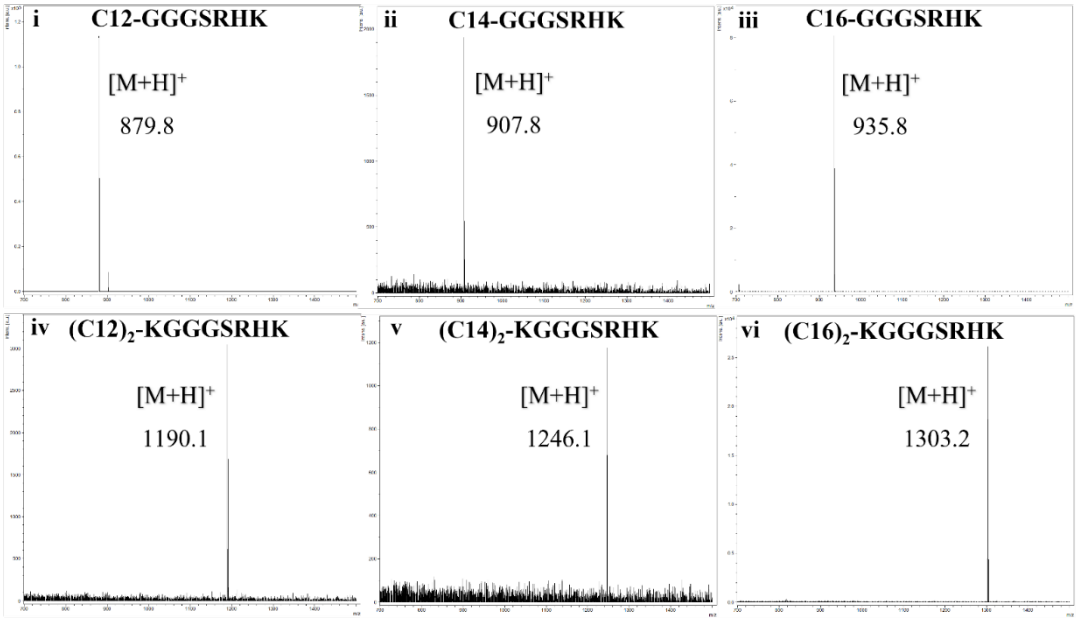


Figure S2. MALDI-TOF-MS results of lipid-GGGS-RHK and $(lipid)_2$-KGGGS-RHK. (**i**) C12-, (**ii**) C14-, (**iii**) C16-GGGS-RHK, (**iv**) $(C12)_2$-, (**v**) $(C14)_2$-, and (**vi**) $(C16)_2$-KGGGS-RHK.

2-2. Conjugation of Q-Tagged chitinase domains by MTG

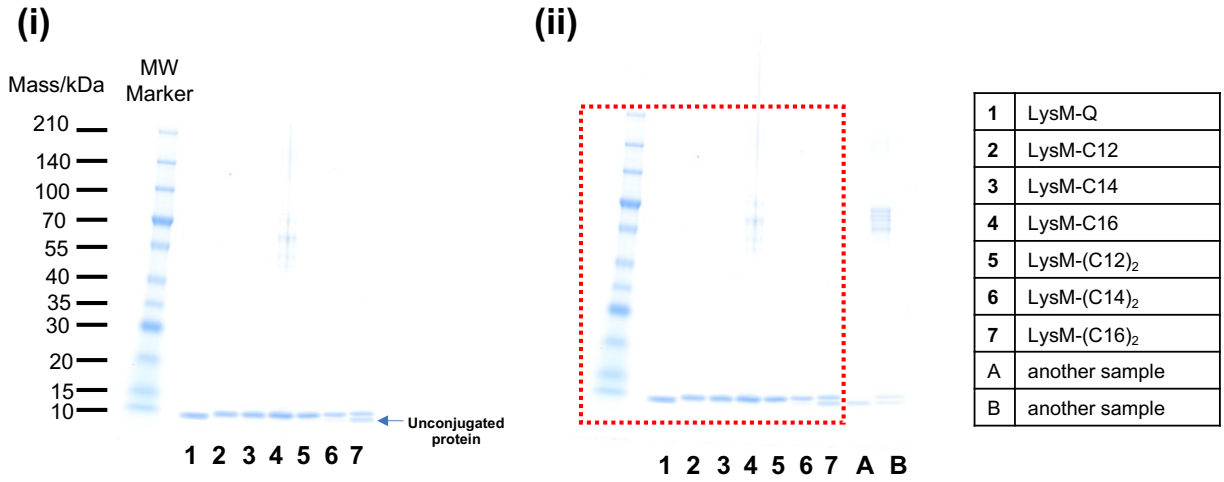


Figure S3. (i) SDS-PAGE analysis of unmodified chitinase and chitinase modified with C12-K, C14-K, C16-K, $(C12)_2$-K, $(C14)_2$-K and $(C16)_2$-K by MTG. All conjugation reactions were carried out with 10 μM Q-tagged chitinase domains, 1% DDM, 10 μM Lipid-K, and 0.1 U/mL MTG in 10 mM Tris-HCl (pH 8.0) at 37 °C for 1 h. (ii) The raw image of SDS-PAGE gel (rectangular box with red dotted line denotes the part used for (i).

2-3. Qualitative results of antifungal activity test

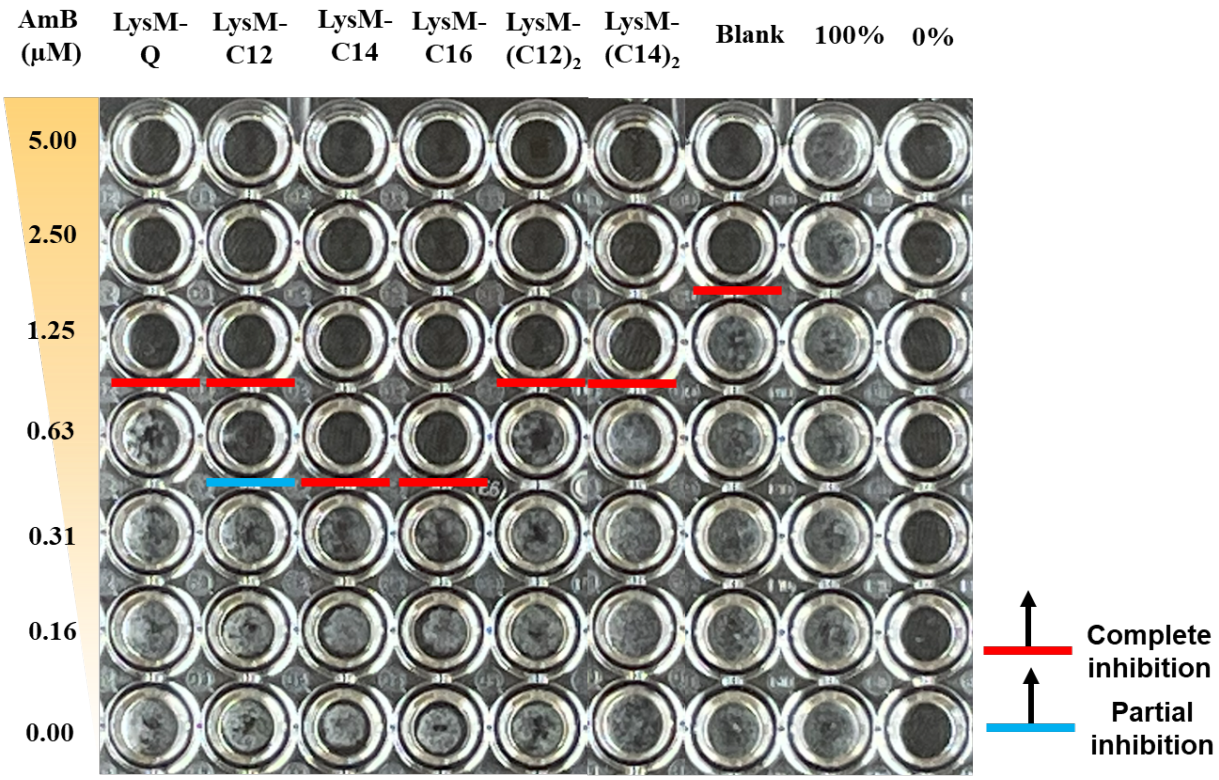


Figure S4. Representative image of a 96-well plate after culturing *T. viride* in the presence of 0−5 μM of AmB with 1 μM of each sample at 60h.

2-4. Antifungal activity test for LysM-C14 and LysM-C16

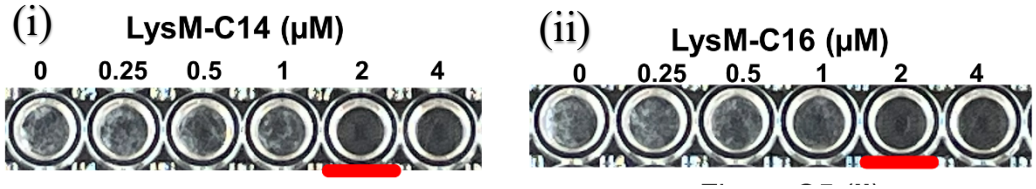


Figure S5. MIC of LysM-C14 (i) and LysM-C16 (ii) in 20 mM NaPi, pH 7.4, 25°C at 60 h.

2-5. FIC index for LysM-C14 and LysM-C16

$$\boldsymbol{FIC\ index} = \frac{A}{MIC_A} + \frac{B}{MIC_B} = FIC_A + FIC_B \qquad (1)$$

The fractional inhibitory concentration (FIC) index was estimated by the above equation (1). "A" refers to the the minimum inhibitory concentration (MIC) value of AmB in combination with LysM-lipid, where "MIC_A" refers to MIC of AmB alone. "B" refers to the MIC value of LysM-lipid in combination with AmB, where "MIC_B" refers to the MIC of LysM-lipid alone. These values added together output the FIC index value, where < 0.5 indicates synergy, 0.5-4 for indifference, and > 4 for antagonism [16].

A. FIC index of LysM-C14

MIC_A	AmB Only	**2.5**	(see Fig. S4)
A	AmB combination	**0.63**	(see Fig. S4)
MIC_B	Protein only	**2**	(see Fig. S5(i))
B	Protein combination	**1**	(see Fig. S4)

$$\boldsymbol{FIC\ index\ of\ AMB\ and\ LysM-C14} = \left(\frac{0.63}{2.5}\right) + \left(\frac{1}{2}\right) = 0.752$$

B. FIC index of LysM-C16

MIC_A	AmB Only	**2.5**	(see Fig. S4)
A	AmB combination	**0.63**	(see Fig. S4)
MIC_B	Protein only	**2**	(see Fig. S5(ii))
B	Protein combination	**1**	(see Fig. S4)

$$\boldsymbol{FIC\ index\ of\ AMB\ and\ LysM-C16} = \left(\frac{0.63}{2.5}\right) + \left(\frac{1}{2}\right) = 0.752$$

2-6. Results of DLS measurements

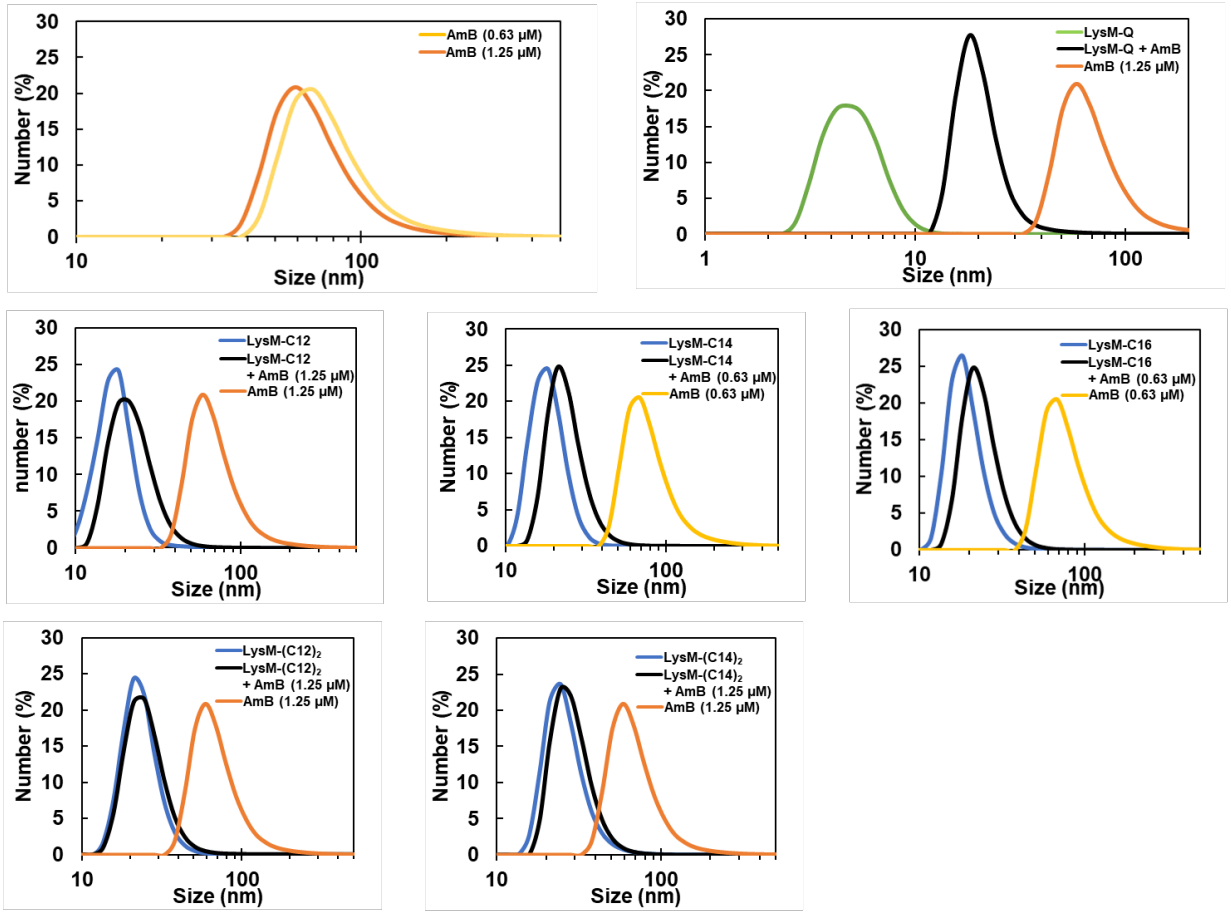
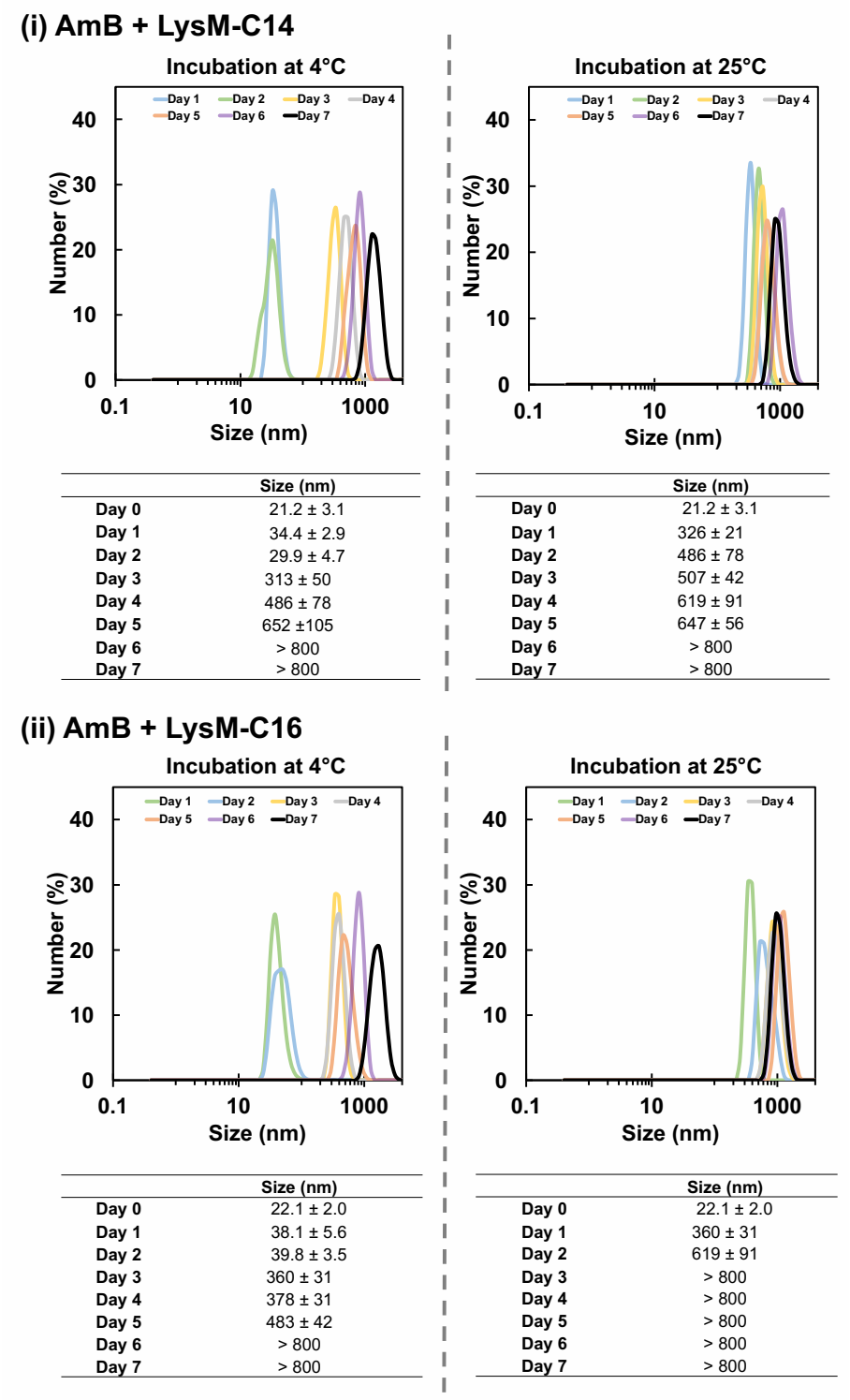


Figure S6. DLS measurements of AmB with LysM-Q or LysM-lipid in 20 mM NaPi, pH 7.4 at 25°C.

2-7. Stability test of the formulation



	Size (nm)
Day 0	21.2 ± 3.1
Day 1	34.4 ± 2.9
Day 2	29.9 ± 4.7
Day 3	313 ± 50
Day 4	486 ± 78
Day 5	652 ±105
Day 6	> 800
Day 7	> 800

	Size (nm)
Day 0	21.2 ± 3.1
Day 1	326 ± 21
Day 2	486 ± 78
Day 3	507 ± 42
Day 4	619 ± 91
Day 5	647 ± 56
Day 6	> 800
Day 7	> 800

	Size (nm)
Day 0	22.1 ± 2.0
Day 1	38.1 ± 5.6
Day 2	39.8 ± 3.5
Day 3	360 ± 31
Day 4	378 ± 31
Day 5	483 ± 42
Day 6	> 800
Day 7	> 800

	Size (nm)
Day 0	22.1 ± 2.0
Day 1	360 ± 31
Day 2	619 ± 91
Day 3	> 800
Day 4	> 800
Day 5	> 800
Day 6	> 800
Day 7	> 800

Figure S7. DLS measurements of AmB with LysM-C14 (i) or LysM-C16 (ii) during incubation at 4°C or 25°C in 20 mM NaPi (pH 7.4).

2-8. Antifungal activity test with the formulation under different storage conditions

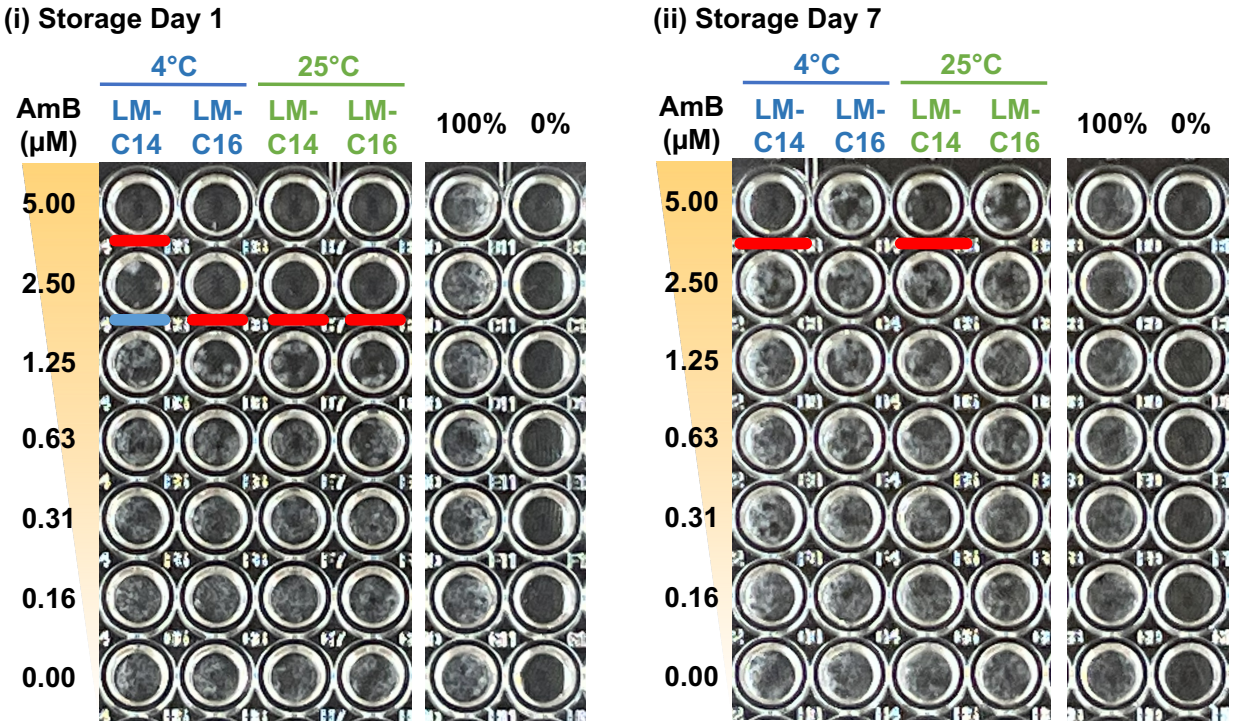


Figure S8. Representative image of a 96-well plate after culturing *T. viride* for 60 h in the presence of 0−5 μM of AmB with 1 μM of LysM-C14 and LysM-C16 under different storage conditions. (i) The formulation after 1 day of storage (Day 1) and (ii) after 7 days of storage (Day 7).

2-9. CLSM analysis of interaction with *T. viride*

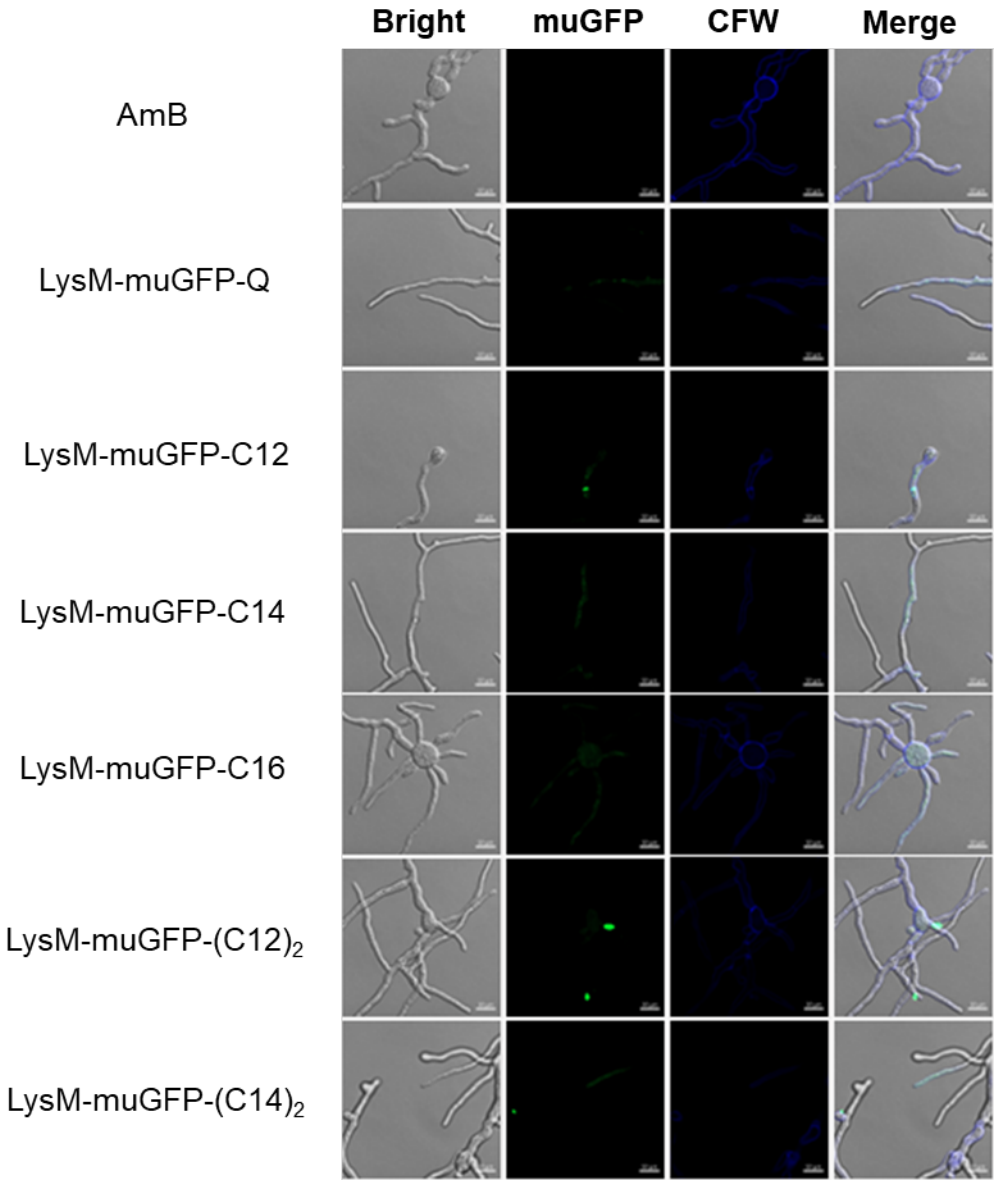


Figure S9. CLSM analyses of LysM-muGFP-Q or LysM-muGFP-lipid and combination without AMB in the presence of *T. viride* hyphae in 20 mM NaPi, pH 7.4, at 25°C (bars: 10 μm).

2-10. Statistical analysis of the binding ability of LysM variants with *T. viride*

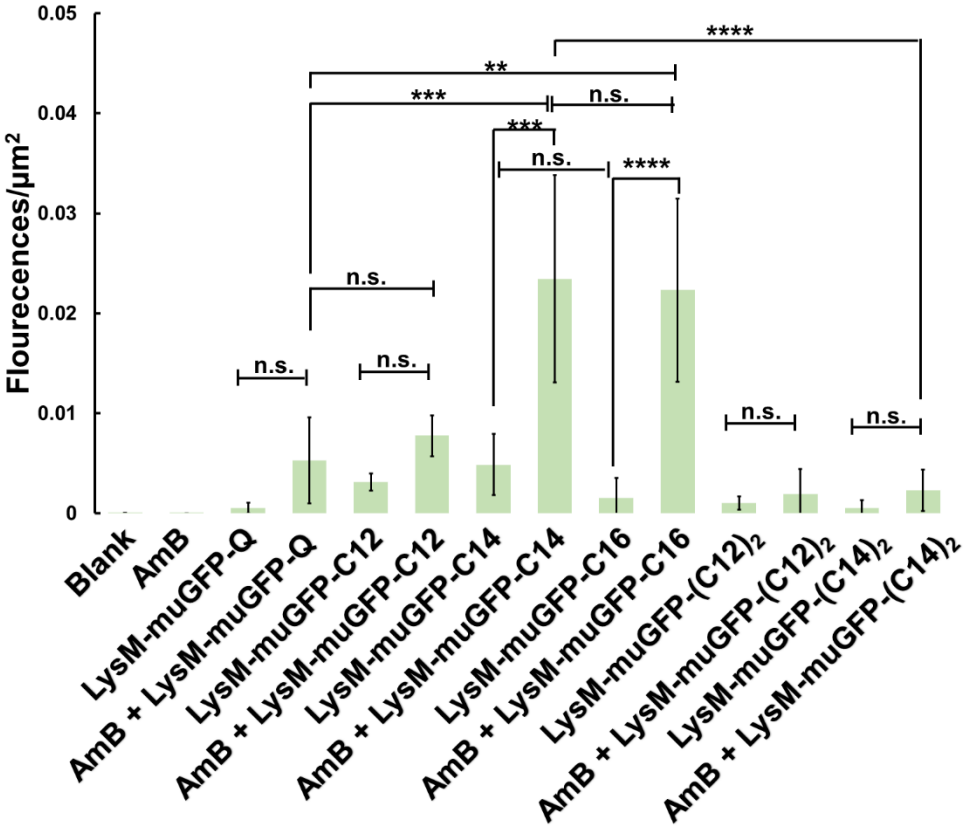


Figure S10. Statistical analysis of the fluorescence imaging results by CLSM (Fig. 3b). $N = 3$; mean ± SE; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. "n.s." indicates not significant ($p > 0.05$) (ANOVA followed by Tukey's multiple comparisons test).

2-11. CLSM analysis of LysM binding activity with α-chitin

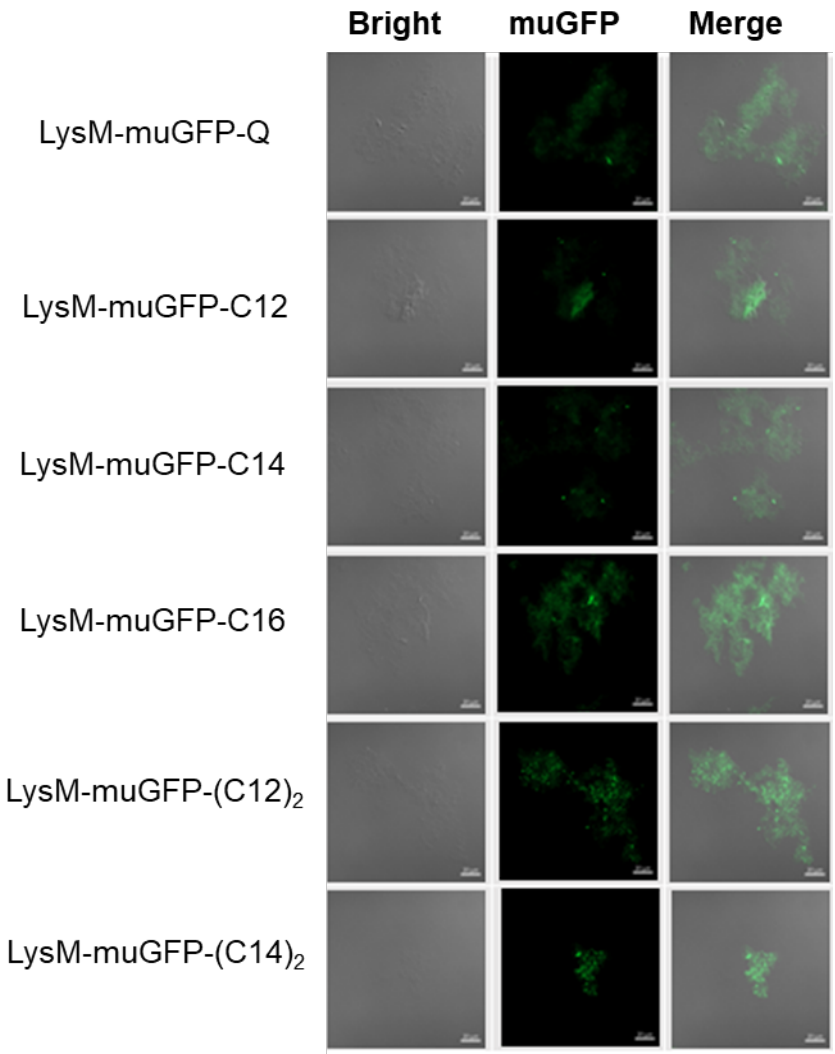


Figure S11. CLSM analyses of LysM-muGFP-Q or LysM-muGFP-lipid in the presence of 0.5% α-chitin in 20 mM NaPi, pH 7.4, at 25°C (bars: 10 μm).

2-12. Cytotoxicity test of LysM-lipids with HeLa cells

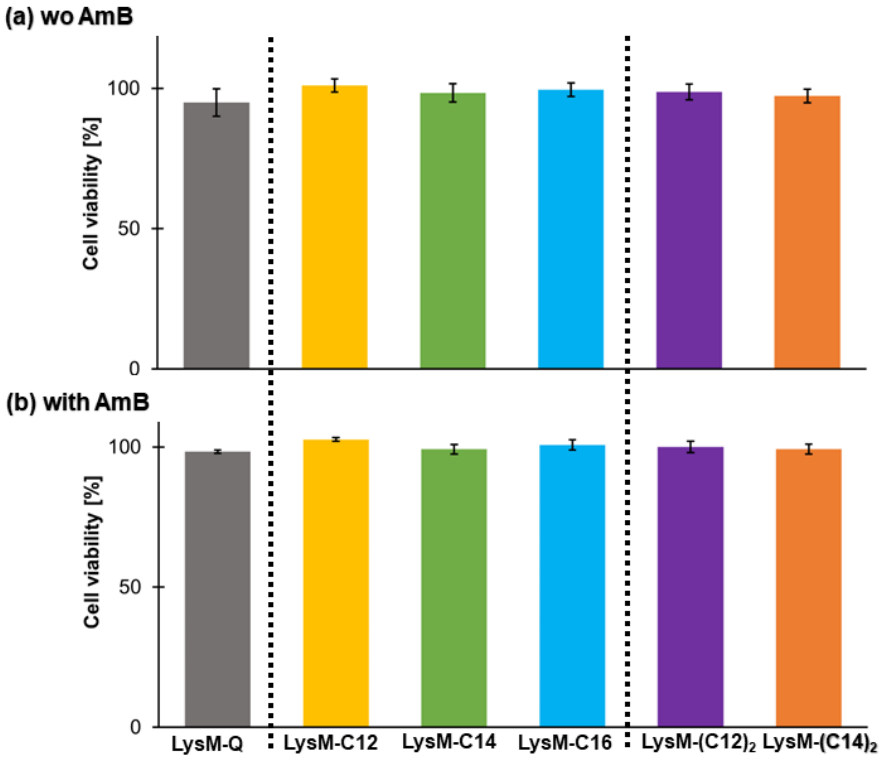


Figure S12. Cell viability of combination chitinase modified and AmB in HeLa cells (5,000 cell/well). Cell viability was quantified by Cell Counting Kit-8 (Dojindo) (A) without AmB and (B) with AmB.