JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

Total Synthesis of Circular Bacteriocins by Butelase 1

Xinya Hemu, Yibo Qiu, Giang K. T. Nguyen, and James P. Tam*

School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, 637551 Singapore

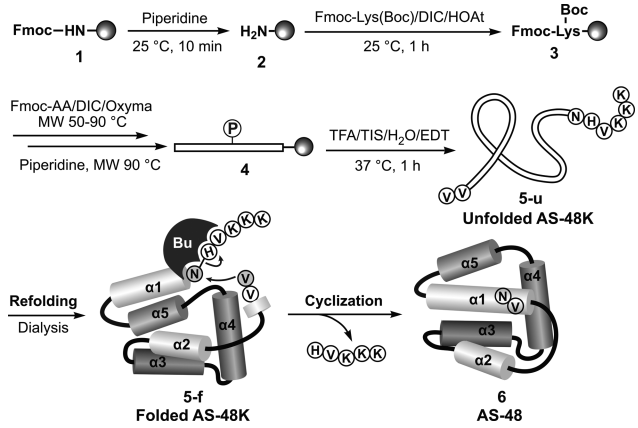
Supporting Information

ABSTRACT: Circular bacteriocins, ranging from 35 to 70 amino acids, are the largest cyclic peptides produced by lactic acid bacteria to suppress growth of other bacteria. Their end-to-end cyclized backbone that enhances molecular stability is an advantage to survive in pasteurization and cooking processes in food preservation, but becomes a disadvantage and challenge in chemical synthesis. They also contain unusually long and highly hydrophobic segments which pose an additional synthetic challenge. Here we report the total synthesis of the three largest circular bacteriocins, AS-48, uberolysin, and garvicin ML, by an efficient chemoenzymatic strategy. A key feature of our synthetic scheme is the use of an Asn-specific butelase-mediated cyclization of their linear precursors, prepared by microwave stepwise synthesis. Antimicrobial assays showed that the AS-48 linear precursor is inactive at concentrations up to 100 μM, whereas the macrocyclic AS-48 is potently active against pathogenic and drug-resistant bacteria, with minimal inhibitory concentrations in a sub-micromolar range.

The circular bacteriocin AS-48 and uberolysin, both 70 residues, are the largest cyclic antimicrobial peptides,[1] and the second only in size to the 78-residue non-antimicrobial pilin.[2] Both of them, together with most members in the circular bacteriocin family, adopt a 4- or 5-helix saposin structure similar to the human antimicrobial peptide granulysin, although they share no sequence homology.[3] In general, circular bacteriocins, including AS-48, are broadly active against bacteria by inducing cell membrane disorganization.[4] AS-48, the best-studied of the group, is a promising candidate as a "natural" food preservative for replacement of chemical preservatives. It is heat-resistant, which ensures its stability in food processing, and sensitive to degradation by digestive proteases, which prevents adverse effects on gut microbiota. More importantly, it is highly potent against many food-borne pathogens, particularly *Listeria*, which can grow at 4 °C and is a leading pathogen causing early termination of pregnancy.[5]

Thus far, cyclic AS-48 is accessible only by expression using *Enterococcus faecalis* in low yield. Its biosynthesis, involving at least 10 genes, remains to be fully elucidated,[6] adding difficulties to its expression by genetic manipulation. Chemical synthesis of a circular bacteriocin has not been reported, because the cyclic backbone and a high content of hydrophobic residues (>50%) pose synthetic challenges (Table 1). A commonly used chemical macrocyclization method is the thia-zip cyclization, a version of native chemical ligation, which requires an N-terminal cysteine and a C-terminal thioester.[7] AS-48 and other circular bacteriocins contain no cysteine, rendering them difficult to synthesize by the thia-zip cyclization but attainable by desulfurization to convert an unwanted Cys from the ligation product to Ala.[8] Our attempt to prepare AS-48 using such an approach (Figure S1) was unsuccessful due to the insolubility of two hydrophobic peptide segments from helices 1 to 3 (Table 1). Several chemoenzymatic methods, using intein and sortase A, have been developed for peptide or protein macrocyclization,[9] but not all are applicable for the total synthesis of native circular bacteriocins. Herein we report a practical and efficient chemoenzymatic synthesis of circular bacteriocins.

Scheme 1. Butelase-Mediated Synthesis of AS-48 6[a]



[a] **1**, Fmoc-PAL-PEG-PS resin; MW, microwave; **4**, protected peptide resin; **5-u** and **5-f** refer to unfolded and folded AS-48K; Bu, butelase 1.

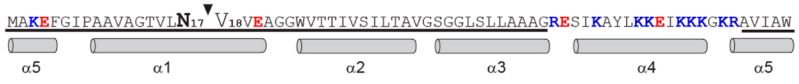
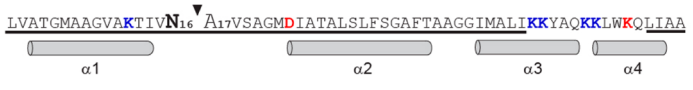
Butelase 1, an Asp/Asn (Asx)-specific ligase, was the key reagent used in our scheme to catalyze peptide cyclization (Scheme 1). Butelase 1 was discovered in our study of naturally occurring cyclic peptides from the leguminous plant *Clitoria ternatea*.[10] It is a highly efficient ligase and displays a broad specificity for the incoming sequence, with the P1″ position accepting any amino acids.[1] The P2″ position favors hydrophobic residues, particularly Leu, Ile, Val, and Cys. Butelase 1 accepts the tripeptide NHV motif as a recognition signal in a precursor and eliminates the HV dipeptide in the ligated product, making the butelase-mediated ligation "traceless" and suitable for total synthesis of natural products. In this regard, butelase 1 has an advantage over sortase A, which results in an extra LPXTG sequence in its ligation product.

To show the generality of butelase-mediated synthesis of circular bacteriocins and the effect of ligation sites, we selected

Received: April 27, 2016
Published: May 20, 2016

DOI: 10.1021/jacs.6b04310
J. Am. Chem. Soc. 2016, 138, 6968–6971

Table 1. Sequences and Ligation Sites of Four Selected Circular Bacteriocins

Peptide	AA	Net-z	Sequence
AS-48	70	+6	
UblA*	70	+3	
GarML*	60	+5	
Ccl	60	+4	

▼, butelase-mediated ligation site. and α, helical regions. Charged residues are colored with blue (K, R) and red (D, E). Hydrophobic regions are underlined. *: helices are predicted by CFSSP software.

four examples: the 5-helical 70-residue AS-48, uberolysin (UblA) the 4-helical 60-residue garvicin ML (GarML), and carnocyclin A (Ccl). Each contains at least one Asn that meets the substrate requirement of a butelase-mediated ligation (Table 1). The N-terminal residue of the linear synthetic precursors **5**, **7**−**11** started with the residue after the selected Asn. For example, AS-48 linear precursors **5** and **7** started with V18 and ended with N17 in helix 1. For comparison, ligation sites of carnocyclin A and garvicin were also in or near helix 1. In contrast, the ligation site of uberolysin was in helix 4. A dipeptide HV was added to the C-terminus of all linear precursor sequences as a recognition signal for butelase-mediated cyclization to give AS-48V **7**, Gar-V **8**, Ccl-V **9**, and UblA-V **11** (Table 2 and Figures S2−S8). We also

Table 2. Summary of Circular Bacteriocin Precursors[a]

	mass (Da)				
precursor	calcd	found	HI	*z*	t_R (min)
AS-48K **5**	7788	7778	0.36	+9	24.5
AS-48V **7**	7403	7408	0.54	+6	25.4
Gar-V **8**	6260	6260	0.87	+5	27.2
Ccl-V **9**	6115	6114	1.04	+4	29.0
UblA-K **10**	7687	7687	0.73	+6	31.8
UblA-V **11**	7302	7298	0.89	+3	>35[b]

[a]Calcd mass = average mass; found mass was determined by MALDI-TOF MS in linear mode. HPLC conditions: 10−95% acetonitrile over 30 min, and followed by 95% for 5 min; C18 column (Phenomenex Aeris PEPTIDE XB-C18, 4.6 mm × 250 mm, 3.6 μm); HI, hydrophobicity index of each peptide, calculated by GPMAW calculator; *z*, net charge; t_R, retention time. [b]UblA-V was not eluted from the C18 column after 35 min using the above gradient conditions. It requires a C4 column with 2-propanol as the elution solvent.

prepared two precursors, AS-48K **5** and UblA-K **10**, with an HVKKK tail for the 5-helix AS-48 and uberolysin, with the expectation to increase the aqueous solubility of these highly hydrophobic linear precursors and to facilitate the butelase-mediated cyclization.

Fmoc chemistry was used to assemble all linear precursors **5**, **7**−**11** on Fmoc-PAL-PEG-PS resin (0.17 mmol/g) using a microwave-assisted synthesizer (CEM Liberty BLUE) with a deprotection step at 90 °C for 0.5 min and a coupling step using DIC/oxyma at 90 °C for 2 min (Scheme 1).[11] With this rapid synthetic protocol, the preparation of the 75-residue precursor AS-48K **5-u** was completed in 6 h. The peptide resin was cleaved by a TFA solution at 37 °C for 1 h to give an unprotected peptide precursor. The diethyl ether precipitated peptide was re-dissolved in 8 M urea and purified by reverse-phase HPLC.

Direct cyclization of the unfolded linear precursor AS-48K **5-u** or AS-48V **7-u** by butelase 1 was unsuccessful, suggesting that AS-48 is protein-like and the butelase-mediated cyclization requires a correctly folded precursor with the N and C termini in close proximity. Thus, linear precursors were refolded by first redissolving in 8 M urea or 6 M guanidine hydrochloride with 5 mM 2-mercaptoethanol at a concentration of 50−100 μM and then folding by direct or stepwise dialysis (see Supporting Information for detailed conditions).

All butelase-mediated cyclizations were performed using an enzyme:peptide molar ratio of 1:100 at pH 6, 37 °C, and monitored by HPLC and MALDL-TOF MS. Using AS-48K as an example, the butelase-mediated cyclization of this 75-residue precursor between a sterically hindered V18 and N17 completes in 1 h (Figure 1A). Analysis by MALDI-TOF mass spectrometry revealed a loss of 638 Da in the ligated product due to the removal of HVKKK and formation of a new peptide bond (Figure 1B). Circular dichroism showed that AS-48K displays an α-helical structure after refolding in a sequentially diluted urea solution, with the helical structure enhanced after cyclization (Figure 1C). The shape and intensity of the mean residue ellipticity of the synthetic AS-48 **6** after cyclization were comparable to the native AS-48 as reported in literature.[12]

To confirm the circular structure and the N17−V18 cyclization site, synthetic AS-48 **6** was subjected to proteolytic digestion to obtain overlapping fragments for LC-MS/MS analysis. A tryptic digested fragment of *m*/*z* 4746.5 Da was analyzed by MS/MS using an Orbitrap analyzer, which confirmed the newly formed peptide bond between N17 and V18 as the ligation site (Figure 2, Figure S9, and Tables S1−S3). In addition, two chymotryptic-digested peptides, A44-Y54 and L55-W70, were sequenced. Together these fragments unambiguously confirmed the cyclic structure of the synthetic AS-48 **6**.

To streamline the proposed chemoenzymatic method without the in-between purification steps, we performed a one-pot synthesis of AS-48 with a single purification step, performed only after the crude AS-48K **5-u** was refolded and cyclized. This one-pot approach required <24 h to complete (6 h for stepwise synthesis, 5 h for refolding, 1 h each for cyclization and

DOI: 10.1021/jacs.6b04310
J. Am. Chem. Soc. 2016, 138, 6968−6971

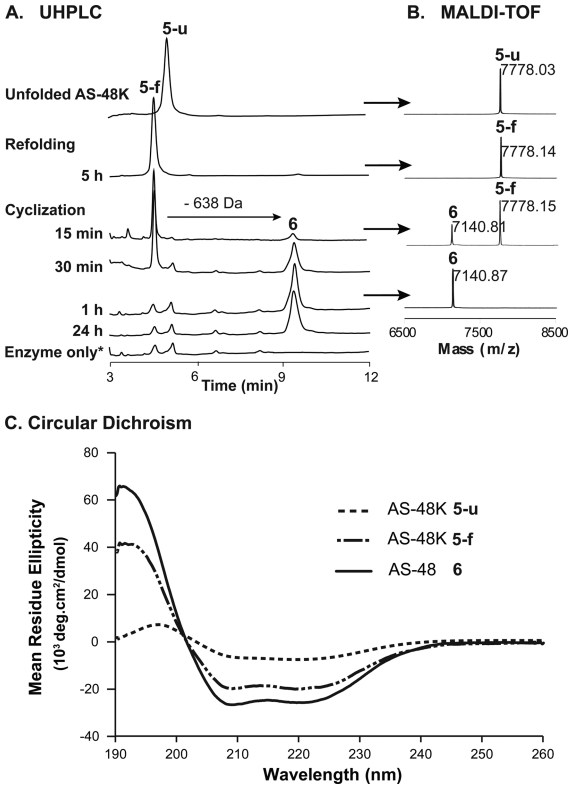


Figure 1. Synthesis and characterization of cyclic AS-48. (A) HPLC monitoring of the reaction. HPLC conditions: buffer A, 0.1% trifluoroacetic acid in Milli-Q water; buffer B, 0.1% TFA in acetonitrile; gradient, 50−60% acetonitrile from 2 to 12 min; column oven, 70 °C; flow rate, 0.3 mL/min. *, impurity peaks from the butelase 1 solution also eluted under this gradient. (B) MALDI-TOF profiles (in linear mode to give m/z in average isotope compositions) of the cyclization mixture at (top to bottom) 0 h, 15 min, 30 min, 1 h, and 24 h. (C) Comparison of circular dichroism spectrum of cyclic AS-48 with those of its precursor AS-48K before and after refolding.

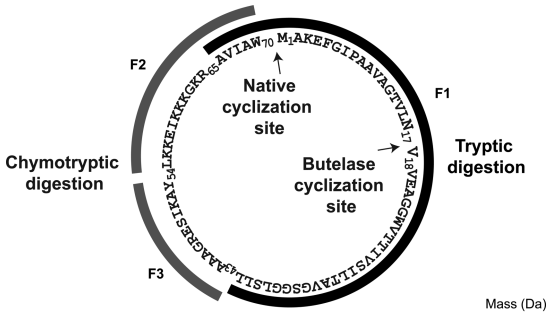


Figure 2. LC-MS/MS analysis of the tryptic and chymotryptic digested AS-48 fragments.

purification) to give 12% yield of AS-48 (2.8 mg purified synthetic AS-48 **6** from 50 mg peptide-resin, Figure S10).

To correlate ligation sites with cyclization efficiency, we compared AS-48K **5-f**, Gar-V **8-f**, and UblA-K **10-f**, which were successfully cyclized in 85−93% yield (Table 3). In garvicin, the ligation site between N16 and A17 is located in the loop between helix 1 and helix 2, whereas in AS-48K, N17 is in helix 1 (Table 1). The cyclization of GarML was complete within 0.5 h (Figure S11), followed by AS-48K in 1 h. Cyclization of the more

Table 3. Generalized Conditions and Yields of Synthetic Circular Bacteriocins[a]

	refolding		cyclization	
peptide	ligation site	time (h)	time (h)	yield (%)
AS-48	α1	5	1	85
GarML	α1	2	0.5	90
UblA	α4	24	24	93

[a]Refolding conditions: all peptides except UblA were dissolved in 8 M urea to the concentration of 0.1 mM and then dialyzed against 100× volume of butelase cyclization buffer. UblA was folded using a stepwise dialysis. It was dissolved in 6 M guanidine hydrochloride to the concentration of 0.1 mM together with 1% Brij 35 and then gradually dialyzed against 8, 6, 4, and 2 M urea for 2 h each. It was finally dialyzed against butelase buffer for 12 h. Cyclization conditions: enzyme:peptide ratio = 1:100 with peptide concentration of 50−100 μM, pH 6, at 37 °C.

hydrophobic UblA-K with a ligation site in helix 4 took 24 h to complete (Figure S12). Our data suggest that butelase-mediated cyclization conducted in the loop region is likely more efficient than that within the helix, and the location of the ligation site could significantly affect the cyclization efficiency.

Another factor that influences butelase-mediated cyclization is the solubility of the precursors. Thus, we investigated the effect of hydrophobicity and the benefit of adding the tripeptide KKK as a solubilizing tail in two analogues of AS-48 and uberolysin without (AS-48V **7**, UblA-V **11**) or with KKK (AS-48K **5**, UblA-K **9**), respectively. By adding the triple Lys, AS-48K **5** and UblA-K **10** were more aqueous soluble, eluted earlier (retention time changed from 25.4 to 24.5 min and >35 to 31.8 min), and gave sharper peaks in HPLC than AS-48V **7** and UblA-V **11**, respectively (Table 2 and Figure S2). Cyclization of the more hydrophobic AS-48V **7** was not complete after 24 h and achieved only 21% yield (Figure S13), whereas the cyclization of AS-48K **5-f** was complete in 1 h with 85% yield. In addition, UblA-V **11** tended to precipitate during the refolding and thus was not able to proceed to cyclization. The triple lysine tail facilitated the aqueous solubility and purification by HPLC and also showed a promoting effect to the butelase-mediated cyclization.

An additional factor affecting our synthesis was found in the synthesis of the highly hydrophobic circular bacteriocin carnocyclin-1. The ligation site locates at the end of helix 1 between N19 and A20-G21. MALDI-TOF MS spectra showed that butelase-mediated cyclization was completed after 2 h, despite its hydrophobic nature (Figure S14). However, we could not quantify the cyclization yield by HPLC because the cyclized carnocyclin was very hydrophobic and difficult to purify using C4 columns, even under elevated temperature. We also tried without success the purification method first reported in the discovery of carnocyclin.[13] Adding a KKK tail to the linear precursor may increase its solubility but cannot remove the problem of solubility of the cyclized product.

Synthetic AS-48 **6** and its linear folded precursor AS-48K **5-f** were examined for their antibacterial activity by radial diffusion assays[14] on seven bacterial strains: *Escherichia coli* and carbapenem-resistant *E. coli* DR23975 (CREC), *Staphylococcus aureus* and methicillin-resistant *S. aureus* DR15686 (MRSA), three lactic acid bacteria strains, *Enterococcus faecium*, *Enterococcus faecalis* V583, and *E. faecalis* OG1RF, closely related to *E. faecalis* S-48, and *Listeria monocytogenes* (Table 4).

Synthetic AS-48 **6** displayed potent inhibitory activity against all tested bacteria, including food-borne *Listeria*, with a minimal

DOI: 10.1021/jacs.6b04310
J. Am. Chem. Soc. 2016, 138, 6968−6971

Table 4. Antimicrobial Activity in MIC of Cyclic AS-48 against Selected Bacteria

bacteria	MIC (μM)	bacteria	MIC (μM)
E. coli	0.42	*E. faecium*	1.18
E. coli DR23975	0.83	*E. faecalis* V583	0.49
S. aureus	0.39	*E. faecalis* OG1RF	1.27
S. aureus DR15686	0.85	*L. monocytogenes*	0.24

inhibitory concentrations (MIC) at a sub-μM range. These results agree with those reported in literature for native AS-48, particularly its high sensitivity against *Listeria*.[6c,15] Importantly, it is active against drug-resistant strains including CREC and MRSA, which further supports that AS-48 acts through a membrane-associated mechanism, different from β-lactam antibiotics. We also observed that the folded linear AS-48K **5** was inactive against all tested bacteria, even at the highest tested concentration of 100 μM. Our result is in agreement with the previous study, which showed that a partially proteolyzed AS-48, with backbone opened at A10/V11 in helix α1, displayed substantially reduced antimicrobial activity.[16] A recent study also showed that AS-48 is active in a dimeric form, where protrusions of Lys13 and Lys40 in helix α1 and α3 to the surface are crucial for dimerization.[17] Thus, the contrasting antimicrobial activity between linear and cyclized AS-48 provides support for the previous findings that macrocyclization maintains the active tertiary structure of AS-48. This is interesting and important, as gut inactivation by protease digestion of circular bacteriocin would protect human microflora from being inhibited and also reduce the chance of developing drug resistance.

In conclusion, we explored the factors for a successful synthesis of highly hydrophobic circular bacteriocins using butelase-mediated cyclization. Important contributing factors include the site for ligation and the hydrophobicity of the linear precursor and the cyclized product, as demonstrated by the satisfactory yields obtained from AS-48K, UBlA-K, and Gal-V. Synthetic AS-48 displayed an antimicrobial activity comparable to that of the native molecules isolated from bacteria. It is broadly active, including against the food-borne pathogens and drug-resistant CREC and MRSA, whereas its linear precursor is inactive. Our proposed chemoenzymatic approach for preparing circular bacteriocins could accelerate the development and application of circular bacteriocins as novel biopreservatives.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b04310.

Experimental procedures for microwave-assisted solid-phase peptide synthesis, refolding, butelase-mediated cyclization, and characterization assays; HPLC and MALDI-TOF MS profiles, including Figures S1−S14 and Tables S1−S3 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*jptam@ntu.edu.sg

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported by the National Research Foundation of Singapore (NRF-CRP8-2011-05) and NTU iFood Research Grant M4081467.080. We thank CEM for preparing the linear peptides by their new model synthesizer Liberty-Blue, National University Hospital (NUH) of Singapore for providing the two drug-resistant strains of *E. coli* and *S. aureus*, and Professor Kimberly Kline from Singapore Centre on Environmental Life Sciences Engineering (SCELSE) for providing the lactic acid bacteria strains.

■ REFERENCES

(1) (a) Galvez, A.; Maqueda, M.; Martinez-Bueno, M.; Valdivia, E. *Res. Microbiol.* **1989**, *140* (1), 57−68. (b) Galvez, A.; Valdivia, E.; Maqueda, M.; Montoya, E. *Microbios* **1985**, *43* (176S), 223−232. (c) Wirawan, R. E.; Swanson, K. M.; Kleffmann, T.; Jack, R. W.; Tagg, J. R. *Microbiology* **2007**, *153* (5), 1619−30.

(2) Eisenbrandt, R.; Kalkum, M.; Lai, E. M.; Lurz, R.; Kado, C. I.; Lanka, E. *J. Biol. Chem.* **1999**, *274* (32), 22548−55.

(3) (a) Liepinsh, E.; Andersson, M.; Ruysschaert, J. M.; Otting, G. *Nat. Struct. Biol.* **1997**, *4* (10), 793−5. (b) Gonzalez, C.; Langdon, G. M.; Bruix, M.; Galvez, A.; Valdivia, E.; Maqueda, M.; Rico, M. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97* (21), 11221−6.

(4) Galvez, A.; Maqueda, M.; Martinez-Bueno, M.; Valdivia, E. *J. Bacteriol.* **1991**, *173* (2), 886−92.

(5) (a) Low, J. C.; Donachie, W. *Vet. J.* **1997**, *153* (1), 9−29. (b) Kaur, S.; Malik, S. V.; Vaidya, V. M.; Barbuddhe, S. B. *J. Appl. Microbiol.* **2007**, *103* (5), 1889−96.

(6) (a) van Belkum, M. J.; Martin-Visscher, L. A.; Vederas, J. C. *Trends Microbiol.* **2011**, *19* (8), 411−8. (b) Martínez-Bueno, M.; Valdivia, E.; Gálvez, A.; Coyette, J.; Maqueda, M. *Mol. Microbiol.* **1998**, *27* (2), 347−358. (c) Maqueda, M.; Galvez, A.; Bueno, M. M.; Sanchez-Barrena, M. J.; Gonzalez, C.; Albert, A.; Rico, M.; Valdivia, E. *Curr. Protein Pept. Sci.* **2004**, *5* (5), 399−416.

(7) (a) Tam, J. P.; Lu, Y. A.; Yu, Q. T. *J. Am. Chem. Soc.* **1999**, *121* (18), 4316−4324. (b) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. *Science* **1994**, *266* (5186), 776−9. (c) Liu, C.-F.; Tam, J. P. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, *91* (14), 6584−6588.

(8) Wan, Q.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2007**, *46* (48), 9248−52.

(9) (a) Tam, J. P.; Wong, C. T. *J. Biol. Chem.* **2012**, *287* (32), 27020−5. (b) Antos, J. M.; Popp, M. W.-L.; Ernst, R.; Chew, G.-L.; Spooner, E.; Ploegh, H. L. *J. Biol. Chem.* **2009**, *284* (23), 16028−16036. (c) Xu, M.-Q.; Evans, T. C., Jr *Methods* **2001**, *24* (3), 257−277.

(10) (a) Nguyen, G. K.; Wang, S.; Qiu, Y.; Hemu, X.; Lian, Y.; Tam, J. P. *Nat. Chem. Biol.* **2014**, *10* (9), 732−8. (b) Cao, Y.; Nguyen, G. K.; Tam, J. P.; Liu, C. F. *Chem. Commun. (Cambridge, U. K.)* **2015**, *51* (97), 17289−92. (c) Nguyen, G. K.; Cao, Y.; Wang, W.; Liu, C. F.; Tam, J. P. *Angew. Chem., Int. Ed.* **2015**, *54* (52), 15694−8.

(11) Collins, J. M.; Porter, K. A.; Singh, S. K.; Vanier, G. S. *Org. Lett.* **2014**, *16* (3), 940−3.

(12) Montalban-Lopez, M.; Spolaore, B.; Pinato, O.; Martinez-Bueno, M.; Valdivia, E.; Maqueda, M.; Fontana, A. *FEBS Lett.* **2008**, *582* (21−22), 3237−42.

(13) Martin-Visscher, L. A.; van Belkum, M. J.; Garneau-Tsodikova, S.; Whittal, R. M.; Zheng, J.; McMullen, L. M.; Vederas, J. C. *Appl. Environ. Microbiol.* **2008**, *74* (15), 4756−4763.

(14) Steinberg, D. A.; Lehrer, R. I. Designer Assays for Antimicrobial Peptides. In *Methods In Molecular Biology*; Shafer, W. M., Ed.; Humana Press: Totowa, NJ, 1997; Vol. 78, pp 169−186.

(15) Mendoza, F.; Maqueda, M.; Galvez, A.; Martinez-Bueno, M.; Valdivia, E. *Appl. Environ. Microbiol.* **1999**, *65* (2), 618−25.

(16) Montalban-Lopez, M.; Martinez-Bueno, M.; Valdivia, E.; Maqueda, M. *Biochimie* **2011**, *93* (3), 549−55.

(17) Cebrian, R.; Martinez-Bueno, M.; Valdivia, E.; Albert, A.; Maqueda, M.; Sanchez-Barrena, M. J. *J. Struct. Biol.* **2015**, *190* (2), 162−72.

DOI: 10.1021/jacs.6b04310
J. Am. Chem. Soc. 2016, 138, 6968−6971