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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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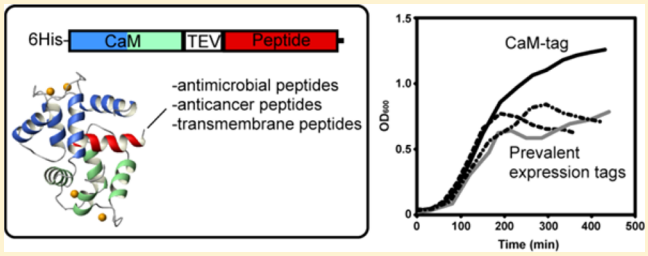
Overexpression of Antimicrobial, Anticancer, and Transmembrane Peptides in *Escherichia coli* through a Calmodulin-Peptide Fusion System

Hiroaki Ishida, Leonard T. Nguyen, Ramamourthy Gopal, Tomoyasu Aizawa,[†] and Hans J. Vogel*

Biochemistry Research Group, Department of Biological Sciences, University of Calgary, Calgary, Alberta T2N 1N4, Canada

Supporting Information

ABSTRACT: In recent years, the increasing number of antibiotic-resistant bacteria has become a serious health concern. Antimicrobial peptides (AMPs) are an important component of the innate immune system of most organisms. A better understanding of their structures and mechanisms of action would lead to the design of more potent and safer AMPs as alternatives for current antibiotics. For detailed investigations, effective recombinant production which allows the facile modification of the amino acid sequence, the introduction of unnatural amino acids, and labeling with stable isotopes for nuclear magnetic resonance (NMR) studies is desired. Several expression strategies have been introduced in previous reports; however, their effectiveness has been limited to a select few AMPs. Here, we have studied calmodulin (CaM) as a more universal carrier protein to express many types of AMPs in *E. coli*. We have discovered that the unique architecture of CaM, consisting of two independent target binding domains with malleable methionine-rich interaction surfaces, can accommodate numerous amino acid sequences containing basic and hydrophobic residues. This effectively masks the toxic antimicrobial activities of many amphipathic AMPs and protects them from degradation during expression and purification. Here, we demonstrate the expression of various AMPs using a CaM-fusion expression system, including melittin, fowlicidin-1, tritrpticin, indolicidin, puroindoline A peptide, magainin II F5W, lactoferrampin B, MIP3α_{51-70}, and human β-defensin 3 (HBD-3), the latter requiring three disulfide bonds for proper folding. In addition, our approach was extended to the transmembrane domain of the cell adhesion protein L-selectin. We propose the use of the CaM-fusion system as a universal approach to express many cationic amphipathic peptides that are normally toxic and would kill the bacterial host cells.



1. INTRODUCTION

Antimicrobial peptides (AMPs) are a large, diverse family of peptides that are essential for the defense mechanism of invertebrates, plants, and animals.[1−3] AMPs have a wide range of activity against bacteria, fungi, viruses, and some parasites,[4,5] and some AMPs have secondary functions in immunomodulation.[6,7] Moreover, many AMPs can also break up bacterial biofilms that are made up of antibiotic-resistant bacteria.[8] In recent years, AMPs have received increased attention as they are potential drug candidates to overcome bacterial resistance to currently prescribed antibiotics.[9] In addition, several AMPs also have anticancer activity, which has further stimulated interest in their properties.[10−13] The main killing mechanism of AMPs is thought to involve the rupture of bacterial membranes, for example, through the formation of transient transmembrane pores. However, many peptides exert their activity through other mechanisms of action such as the inhibition of cell wall synthesis or other enzymatic activities, interfering with DNA/RNA/protein synthesis, or inhibiting essential intracellular proteins.[14,15] Therefore, detailed structural information regarding the interactions between AMPs and their distinct molecular targets will undoubtedly improve our understanding of their various functions.[16−18]

NMR is currently the most powerful technique to characterize the structural and dynamic properties of such peptides in complex with their potential molecular targets such as micelles and other membrane mimetics or nucleotides and proteins. Recently, nanodiscs of various sizes, which are much better mimetics for biological membranes than micelles, have been designed, and they were successfully used to study membrane proteins by NMR spectroscopy.[19−21] In order to apply advanced NMR techniques in such systems, relatively large amounts of partially or fully isotope-labeled proteins and peptides containing ^{13}C, ^{15}N, and ^{2}H in the case of large complexes, are desired.[22] The chemical synthesis of isotope-labeled peptides is often very costly and not a practical choice. Therefore, a number of methods for the production of recombinant AMPs in bacteria have been developed.[23−27] The main challenge associated with the expression of AMPs is their lethal activity toward the host bacteria. Additionally, the vast majority of AMPs are highly basic and therefore susceptible to degradation by endogenous proteases. A key element for the success for expression of AMPs appears to be the use of a

Received: June 30, 2016
Published: August 9, 2016

DOI: 10.1021/jacs.6b06781
J. Am. Chem. Soc. 2016, 138, 11318−11326

carrier protein that is expressed together with an AMP as a fusion partner. The anticipated role of this carrier protein would be to mask the toxicity of AMPs and to also afford protection from proteolytic degradation. Alternatively, AMP-fusion proteins have been targeted toward inclusion bodies where they are thought to be more stable and not harm the bacterial cell.[28] Although some individual successes have been reported, a bacterial expression system that could be used widely for producing AMPs would facilitate many future studies. For example, just like isotope-labeling of proteins, the ability to routinely isotope-label peptides would give many new opportunities for NMR structural and dynamic studies. When screening some proteins for binding AMPs, we discovered that calmodulin (CaM) could bind these peptides in a manner that resembled its interaction with peptides encompassing the CaM-binding domains of target proteins (*vide infra*). Hence, we elected to develop a CaM-fusion protein system.

Calmodulin (CaM) is a ubiquitous eukaryotic calcium-sensor protein which can interact with various proteins and transmit calcium signals downstream to initiate various physiological events.[29,30] The 148-residue protein consists of two similar globular domains (the N- and C-lobes) that are connected by a highly flexible joint. This allows the two domains to move freely and independently in solution, which is thought to be responsible for CaM's promiscuous target recognition properties.[31] Indeed, CaM-binding peptides are highly variable in amino acid sequence.[32,33] Interestingly, CaM-binding peptides derived from distinct target proteins do not share sequence homology, but they do have very similar properties when compared to AMPs (Figure 1a).

Typically, both are linear peptides that contain many Arg and Lys residues, resulting in a high net positive charge (pI > 10). This is thought to be important for the initial attraction of the peptide to a negatively charged surface such as a bacterial membrane or CaM. They also contain a high content of hydrophobic residues, with Trp residues in particular appearing frequently in both types of peptides (Figure 1a). These characteristics give both types of peptides the propensity to form an amphipathic conformation when binding to a membrane surface or to CaM.[16,30] Indeed, such interactions have already been reported for CaM and the cytotoxic peptides mastoparan-X[35] and mellitin,[36] and the AMP indolicidin.[37]

Although a CaM fusion-protein has previously been reported for facilitating efficient purification of proteins,[38] it has not been studied before whether it can be used to overcome the toxicity and stability problems described above. Our expression system has resulted in the successful expression and purification of many known AMPs including melittin,[39] fowlicidin-1,[40] indolicidin,[41] tritrpticin,[42] PuroA,[43] magainin II F5W,[44] lactoferrampin B,[45,46] and MIP3α_{51-70}.[47] These all belong to the helical and turn-like classes of AMPs. Moreover, several of these peptides are known to have anticancer activities. Furthermore, we demonstrate the purification of a larger antimicrobial peptide, human β-defensin 3 (HBD-3),[48] that is correctly folded with three intramolecular disulfide bonds using our CaM-fusion system.

We have extended our approach to express and purify a transmembrane domain from the cell adhesion molecule L-selectin. The overexpression of transmembrane protein domains in host bacteria is often associated with toxicity and low solubility, resulting in insufficient yields that have impeded structural studies. Similarly to the AMPs, transmembrane domains frequently contain basic residues that flank hydrophobic transmembrane residues, making them suitable for CaM association. Using the CaM-fusion tag, we also obtained isotope-labeled L-selectin transmembrane peptide for advanced NMR studies, a construct that could not be expressed with other expression tags.

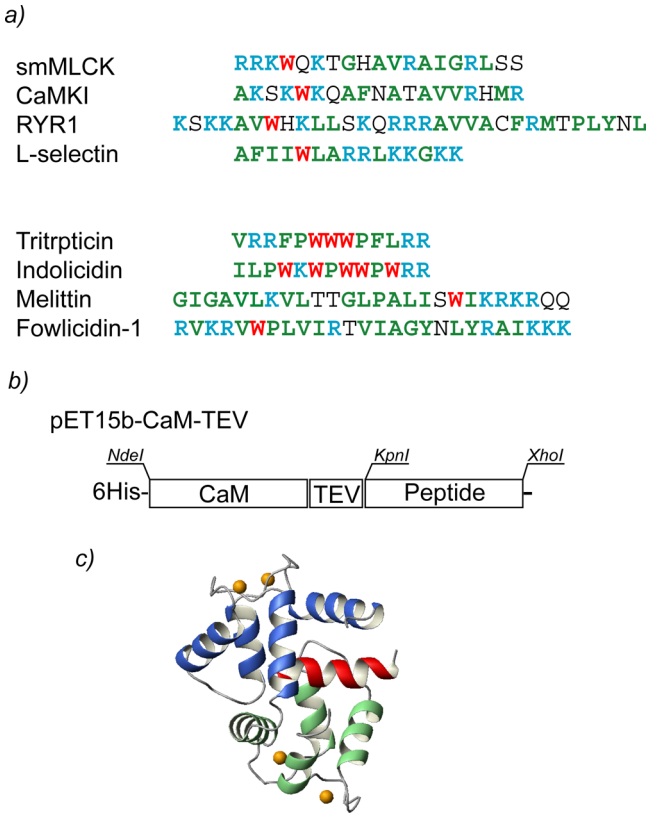


Figure 1. (a) Amino acid sequences of CaM binding peptides derived from various target proteins (top) and various AMPs (bottom). The Trp residues that are frequently identified in both types of peptides are highlighted in red. The basic and hydrophobic residues are shown in cyan and green, respectively. (b) Schematic representation of the CaM-peptide fusion vector. (c) A ribbon structure showing Ca^{2+}-CaM in complex with the L-selectin peptide (residues 349−363, PDB code: 2LGF).[34] The N- and C-domains of CaM are shown in blue and green, respectively, and wrap around the α-helical L-selectin peptide shown in red. The four calcium ions are indicated as gold spheres.

2. EXPERIMENTAL PROCEDURES

Peptide Expression and Purification. All the primers used to generate CaM-peptide fusion expression systems (Figure 1b) are listed in the Supporting Information. The gene for chicken calmodulin was amplified from plasmid pCCM0[49] by standard PCR with primers which also contained a tobacco etch virus (TEV) protease recognition site followed by *Kpn*I and *Xho*I sites and *Pfu* DNA polymerase (Thermo Scientific). The amplified gene was then subcloned into the pET15b vector (Invitrogen) using *Nde*I and *Xho*I sites and T4 DNA ligase (Thermo Scientific) to create the CaM-TEV vector. Using this vector as a template, the gene for CaM followed by the TEV site was amplified by standard PCR with primers which contained the codon-optimized gene for the peptide of interest. These peptides include the following: melittin, tritrpticin, and indolicidin. The genes were again subcloned into the pET15b vector using *Nde*I and *Xho*I sites to generate the CaM-fusion expression vectors for these peptides. The codon-optimized gene for the transmembrane domain of L-selectin (residues 318−372, L-sel$_{318-372}$) was chemically synthesized (GeneArt) and subcloned into the CaM-TEV vector using *Kpn*I and *Xho*I sites to generate the expression vector for the L-sel$_{318-372}$. To produce

DOI: 10.1021/jacs.6b06781
J. Am. Chem. Soc. 2016, 138, 11318−11326

expression vectors coding CaM-fused fowlicidin-1, magainin 2 F5W, MIP3α_{51-70}, and lactoferrampin B, we employed the inverse PCR technique using the CaM-melittin or CaM-tritrpticin vector as a template (for primer design, see Supporting Information Table S1). Prior to transformation, the template vector was digested with Dpn I (Thermo Scientific). To generate the expression vector for HBD-3, the gene encoding this peptide was chemically synthesized and subcloned into the CaM vector using the *Kpn*I and *Xho*I sites. To generate expression plasmids for the N-terminal His_6-tagged melittin, L-sel$_{318-372}$, and tritrpticin without a CaM fusion partner, the PCR amplified genes were subcloned into pET15b vectors using the *Nde*I and *Xho*I sites. The expression vectors for the KSI-fused melittin, L-sel$_{318-372}$, and tritrpticin were generated as previously described.[50] To generate the expression plasmids for the glutathione S-transferase (GST)-fused melittin and L-sel$_{318-372}$, the PCR amplified genes were subcloned into the pGEX-6P-1 vector (GE Healthcare) using the *Bam*HI and *Xho*I sites. The recombinant plasmid for HBD-3 was transformed into competent *E. coli* strain Origami B(DE3) to promote the correct formation of its three internal disulfide bonds. All other recombinant plasmids were transformed into competent *E. coli* strain BL21(DE3).

E. coli cells were grown in Luria broth (LB) media at 37 °C with 100 μg/L ampicillin. For the preparation of uniformly ^{15}N- and ^{15}N,^{13}C-labeled peptides, the cells were grown in M9 medium containing 0.5 g/L $^{15}NH_4Cl$ and 3g/L $^{13}C_6$-glucose (or unlabeled glucose) (for recipe see Supporting Information). Fusion proteins were induced at an OD_{600} of ∼0.7 with 0.5−1.0 mM IPTG. After 4 h of induction at 37 °C, cells were harvested by centrifugation (7800 × *g*, 20 min). The cell pellet was resuspended in 30 mL of IMAC binding buffer (20 mM Tris/HCl, 150 mM NaCl, pH 8.0) and lysed by three passes through a French press. After a high-speed centrifugation of the lysate (39 000 × g, 40 min), the supernatant was applied onto an IMAC column (GE Healthcare) equilibrated with the IMAC binding buffer. The column was washed extensively (at least 10× column volume) with wash buffer (20 mM Tris/HCl, 150 mM NaCl, 50 mM imidazole, pH 8.0), and the fusion protein was eluted with elution buffer (20 mM Tris/HCl, 150 mM NaCl, 300 mM imidazole, pH 8.0). Peak fractions were detected by A_{280} and/or the Bio-Rad protein assay (Bio-Rad Laboratories). The peak fractions were pooled and dialyzed overnight in 4 L of 20 mM Tris/HCl, 100 mM NaCl pH 8.0 at 4 °C. After the addition of 0.5 mM EDTA and 1 mM dithiothreitol (DTT), the CaM-peptide protein construct was subjected to TEV protease digestion at 30 °C for 1 h. TEV protease was expressed and purified from the pRK793 plasmid (Addgene) as previously described.[51,52] To preserve the disulfide bonds in the CaM-HBD-3 fusion, the digestion buffer contained a mixture of 30 mM reduced glutathione and 3 mM oxidized glutathione instead of DTT.

Prior to reverse-phase high-performance liquid chromatography (RP-HPLC), the digested mixtures were acidified to pH 3 with trifluoroacetic acid (TFA, usually 0.1%). The peptides were purified from a Cosmosil 5C_{18} AR-300 column (Nacalai Tesque Inc., Kyoto, Japan) running a gradient from buffer A (0.05% TFA in filtered water) to buffer B (0.045% TFA in HPLC-grade acetonitrile). Relevant fractions containing the peptide of interest were collected and lyophilized. Peptide purity was confirmed by Coomassie brilliant blue staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), RP-HPLC, and subsequently by MALDI-TOF mass spectrometry, which was carried out at the Alberta Proteomics and Mass Spectrometry Facility (Edmonton, AB, Canada).

Surface Plasmon Resonance (SPR). The binding between Ca^{2+}-CaM and several AMPs was evaluated by SPR using a BIAcore X100 instrument (GE Healthcare). For these experiments, a CaM variant (Cys-CaM) with an extra N-terminal Cys residue was used. This CaM was expressed and purified from *E. coli* BL21(DE3) as described previously.[53] The peptides used here were purchased as synthetic peptides with >95% purity from Genscript (San Diego, CA).

Cys-CaM was immobilized via thiol-coupling onto a CM5 sensor chip (GE Healthcare). The running buffer contained 10 mM Tris-HCl pH 7.5, 150 mM KCl, 1 mM $CaCl_2$, and 0.005% (v/v) Tween-20. Five concentrations of peptide solutions were prepared for each cycle, starting at 167 nM and continuing with 3-fold dilutions after. Peptides were injected at a flow rate of 30 μL/min with a contact time of 1 min at 25 °C. The BIAevaluation software 2.0 (GE Healthcare) was used to process the SPR sensorgrams and for curve-fitting to obtain the dissociation constants (K_D).

NMR Experiments. All heteronuclear single quantum correlation (HSQC) NMR spectra were acquired at 30 or 37 °C on a Bruker Avance 700 MHz NMR spectrometer equipped with a cryoprobe with a single axis *z*-gradient. The NMR sample for the tritrpticin peptide contained ∼0.5 mM ^{15}N-labeled tritrpticin, 100 mM n-dodecylphosphocholine (DPC), pH 4.3. The NMR sample for the fowlicidin peptide contained 0.15 mM ^{15}N-labeled fowlicidin-1, 20 mM Bis-Tris (pH 6.9), 100 mM KCl, and 2 mM $CaCl_2$. For the NMR sample of the CaM-fowlicidin-1 peptide complex, the sample contained an additional 0.2 mM unlabeled CaM. The NMR sample of L-sel$_{318-372}$ contained 0.5 mM ^{15}N-labeled peptide in 20 mM sodium phosphate (pH 6.5), 100 mM KCl, and 300 mM 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC). The NMR sample of melittin contained ∼6.5 mM ^{15}N-labeled melittin in water. All NMR samples contained 10% D_2O, 0.5 mM 2,2-dimethyl-2-silapentanesulfonic acid (DSS), and 0.03% NaN_3. Chemical shifts for ^1H and ^{15}N in all spectra were referenced using DSS. All HSQC spectra were processed using NMRPipe[54] and analyzed with NMRView.[55] Molecular images were created with MOLMOL.[56]

The diffusion NMR experiments were carried out on a Bruker Avance 500 MHz NMR spectrometer equipped with a ^1H, ^{15}N, ^{13}C TXI probe with triple axis gradients. There were 64 experiments with increasing gradient strength acquired and analyzed as previously described.[57] The NMR samples for the diffusion experiments contained 0.5−1.0 mM protein, 100 mM KCl, 10 mM $CaCl_2$, and 0.01% 1,4-dioxane in 99.96% D_2O at pH 7.2 ± 0.1. The samples for CaM-fowlicidin-1, CaM-tritripticin, and CaM-indolicidin required an extra 200 mM KCl to prevent the formation of intermolecular interactions.

3. RESULTS AND DISCUSSION

CaM Binds to Several AMPs with High Affinity. To evaluate whether calmodulin is a suitable fusion partner for the recombinant production of toxic peptides in *E. coli*, we first determined its potential binding abilities with AMPs using SPR. In general, the two domains of Ca^{2+}-CaM wrap around its peptide partner,[31] which would potentially mask the harmful effects of the peptide during expression. Previously, we have reported the solution NMR structure of an L-selectin peptide complexed with Ca^{2+}-CaM (Figure 1c), in which the N- and C-lobes of CaM bind to the α-helical peptide, covering a large portion of its sequence.[34] This interaction may also protect the peptide from degradation by host proteases throughout the intracellular expression and subsequent purification.

Figure 2 shows the SPR sensorgrams obtained for several peptides passed over immobilized Cys-CaM, with the calculated K_D values summarized in Table 1.

The dissociation constants for the peptides tested range between 10^{-8} and 10^{-6} M, showing fairly strong affinities. It appears that the peptides with a higher PI value resulted in higher affinity, which was expected as the positive charges are important to associate with the negatively charged surface residues of CaM. In our recent report, the L-selectin transmembrane peptide also associated strongly with Ca^{2+}-CaM with nanomolar affinity.[34] Of the peptides tested, melittin, fowlicidin-1, and magainin F5W become α-helical upon binding to membranes, which is characteristic of most CaM-binding peptides. On the other hand, peptides such as tritrpticin and indolicidin do not form α-helices in membranes,[16] and CD spectroscopy data also showed that these two peptides did not form α-helices when bound to Ca^{2+}-CaM (data not shown).

DOI: 10.1021/jacs.6b06781
J. Am. Chem. Soc. 2016, 138, 11318−11326

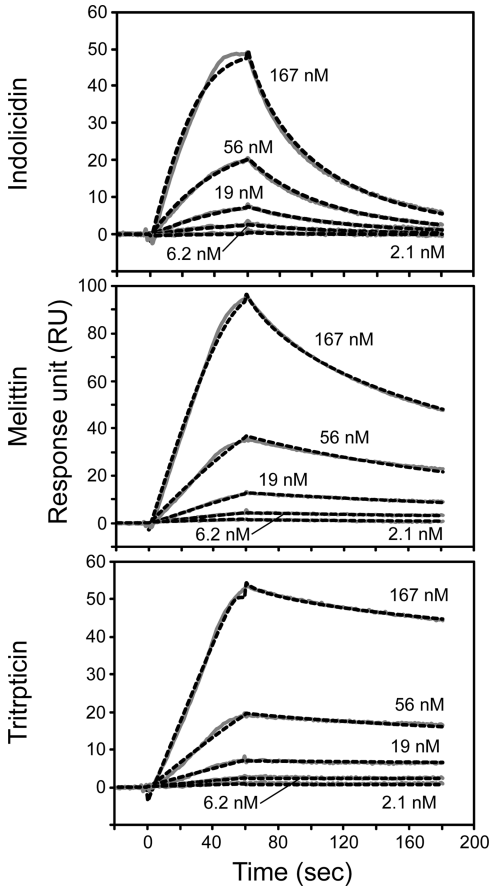


Figure 2. SPR measurements of the binding of AMPs to Ca^{2+}-CaM. Sensorgrams and fittings are shown in solid gray lines and dashed black lines, respectively. Indolicidin, melittin, and tritrpticin peptides in the concentrations of 2.1, 6.2, 19, 56, and 167 nM were injected.

Table 1. Determination of Binding Affinities between AMPs and Ca^{2+}-CaM by Surface Plasmon Resonance

peptide[a]	sequence	pI	K_D (M)
tritrpticin	VRRFPWWWPFLRR	12.5	$(1.70 \pm 0.12) \times 10^{-8}$
indolicidin	ILPWKWPWWPWRR	12.0	$(2.11 \pm 0.08) \times 10^{-7}$
melittin	GIGAVLKVLTTGLPA LISWIKRKRQQ	12.0	$(3.50 \pm 0.18) \times 10^{-8}$
magainin II F5W	GIGKWLHSAKKFGKA FVGEIMNS	10.0	$(1.52 \pm 0.06) \times 10^{-6}$
puroA	FPVTWKWWKWWKG	10.3	$(4.27 \pm 0.23) \times 10^{-7}$
fowlicidin-1	RVKRVWPLVIRTVIA GYNLYRAIKKK	11.6	$(4.27 \pm 0.23) \times 10^{-7}$

[a]SPR sensorgrams for lactoferrampin B; MIP3α_{51-70} and HBD3 could not be analyzed as these peptides also interacted with the reference cell surface.

Therefore, their strong interactions with CaM indicates that the propensity for an α-helical structure is not an absolute requirement for binding to CaM. Rather, the high content of hydrophobic and positively charged residues, and the ability to form an amphipathic structure, appears to be sufficient.

HBD-3 represents a different type of AMP. It is larger at 45 residues, and this miniprotein is already folded in aqueous solution, with a structure that features three internal disulfide bonds that hold a three-stranded β-sheet to a short α-helix.[48] SPR experiments were attempted with our purified HBD-3 injected over the CaM-immobilized sensor chip; however, the strong association of HBD-3 to the reference cell prohibited the analysis of these results. Instead, to confirm binding, NMR experiments were performed using ^{15}N-labeled Ca^{2+}-CaM. Upon addition of HBD-3, many of the HSQC peaks disappeared, indicating intermediate exchange which is associated with micromolar affinity between these two proteins.

We also used NMR spectroscopy to investigate the overall structural properties of CaM-peptide fusion proteins. The hydrodynamic radii (R_h) of CaM-peptide fusions as determined by diffusion NMR experiments are listed in Table 2.

Table 2. Hydrodynamic Radii (R_h's) of CaM-Tag Fused with Various AMPs

protein	no. AA	R_h (Å)
CaM	148	25.3 ± 0.1
His_6-CaM-TEV	179	26.2 ± 0.2
CaM-melittin	205	25.1 ± 0.2
CaM-tritrpticin	191	28.4 ± 0.1
CaM-fowlicidin-1	204	24.0 ± 0.4
CaM-indolicidin	191	28.6 ± 0.2

As expected, the R_h value of Ca^{2+}-CaM attached to the His_6-tag, and the TEV protease cleavage site (His_6-CaM-TEV), appears to be slightly larger than that of Ca^{2+}-CaM. The R_h values of CaM-melittin and CaM-fowlicidin-1 fusions show substantially smaller R_h values than that of His_6-CaM-TEV, indicating that these fusion proteins form a compact structure in which the N- and C-domains of CaM are collapsed onto the peptide. This observation is consistent with other CaM-target complexes.[57] On the other hand, the shorter peptides such as tritripticin and indolicidin show a larger R_h than His_6-CaM-TEV, indicating that these fusion proteins form an extended conformation where only one lobe of CaM, most likely the C-domain, associates with the fused peptide. This observation implies that for some short peptides a single domain of CaM may be sufficient as a fusion partner to mask their toxicity. Next, we monitored the structural changes of one of the peptides upon the addition of CaM. The HSQC spectra in Figure 3 show the amide peaks of our purified ^{13}C,^{15}N-labeled fowlicidin-1 in aqueous solution and upon the addition of unlabeled Ca^{2+}-CaM.

The peptide experiences a large conformational change from a random coil structure where all the signals appear around 8 ppm in the proton dimension to a more spread out structured conformation. A series of three-dimensional backbone NMR experiments were also acquired, and the chemical shift index values from the assigned Cα and CO chemical shifts of CaM-bound fowlicidin-1 indicate that it has an α-helical conformation throughout most of the peptide (Figure 3c). From the chemical shift changes in the HSQC spectrum of ^{15}N-labeled Ca^{2+}-CaM attached to fowlicidin-1, it is clear that both domains of CaM are involved in the interaction (Figure S1). The HSQC signals of ^{15}N-fowlicidin-1 peptide bound to unlabeled Ca^{2+}-CaM can be overlaid well on the HSQC spectrum of ^{15}N-labeled Ca^{2+}-CaM-fowlicidin-1 fusion protein, indicating that the structures of the complex and the fusion protein are essentially the same. Taken together with the SPR and the diffusion NMR data, we conclude that Ca^{2+}-CaM associates with the fused fowlicidin-1 peptide in a similar fashion as other CaM-target peptide interactions, where the two domains of CaM are collapsed onto the target peptide and cover most of the peptide. We hypothesize that this mode of

DOI: 10.1021/jacs.6b06781
J. Am. Chem. Soc. 2016, 138, 11318−11326

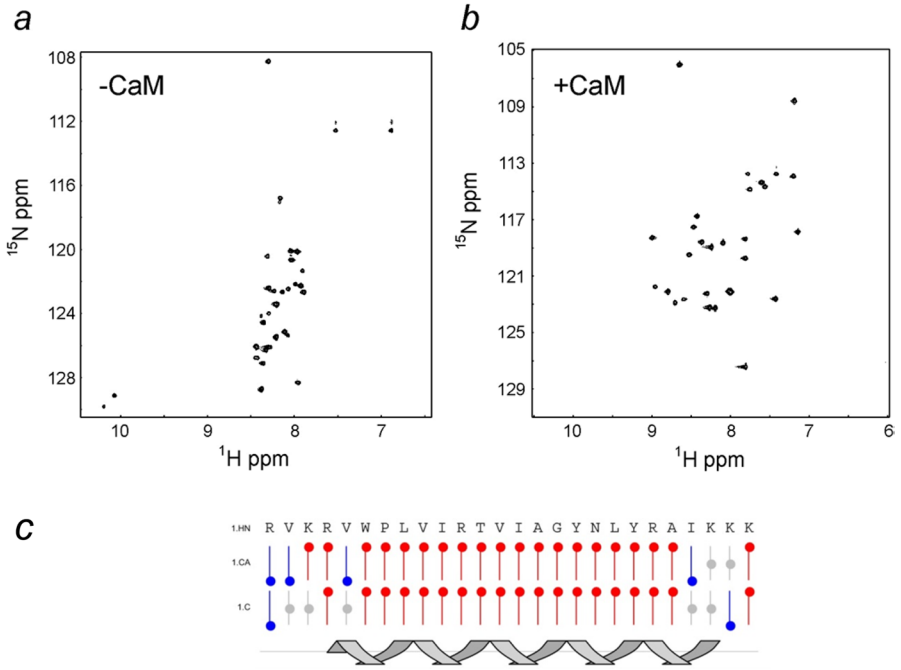


Figure 3. ^{1}H,^{15}N-HSQC spectra of ^{15}N-labeled fowlicidin-1 peptide (a) without and (b) with Ca^{2+}-CaM. (c) Chemical shift index (CSI) for the assigned Cα and CO atoms of ^{13}C,^{15}N-labeled fowlicidin-1 bound to Ca^{2+}-CaM. The region with consecutive positive CSI values (colored in red) represents an α-helical conformation.

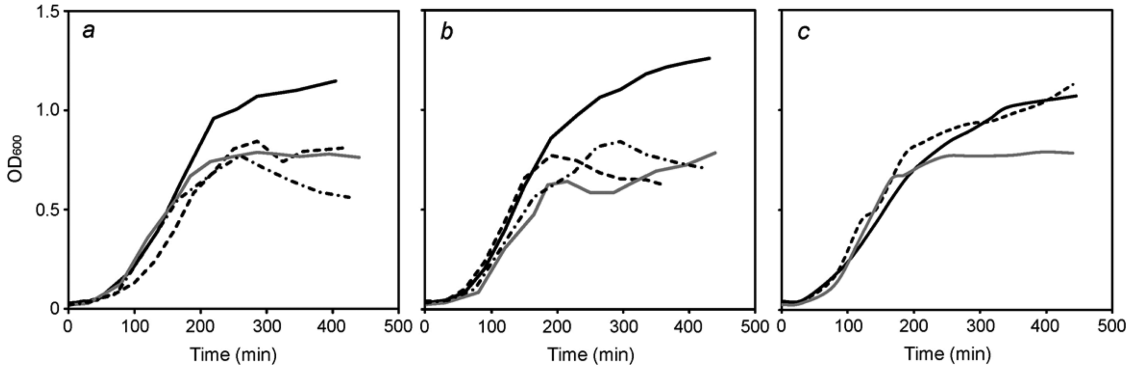


Figure 4. Effect of IPTG-induced peptide expression on the growth of *E. coli* BL21 host cells. (a) Melittin, (b) L-sel$_{318-372}$, and (c) tritrpticin were expressed with (His)$_6$-tag (gray line), KSI (dashed line), GST (dot-dashed line), or CaM (solid line). Cells were grown in 5 mL of LB medium at 37 °C and induced with 0.5 mM IPTG at an $OD_{600} \sim 0.7$.

binding can mask the toxicity of the fowlicidin-1 peptide during expression.

Expression of CaM-Fused AMPs Masks Their Activity. In order to demonstrate the effects of the CaM fusion partner on the viability of host *E. coli*, we monitored the growth of *E. coli* cultures that express different AMPs fused with an N-terminal CaM-tag and other commonly used fusion expression tags. Figure 4 shows representative growth curves for *E. coli* cells in LB media that express either melittin, L-sel$_{318-372}$, or tritrpticin with these different tags.

The IPTG-induced expression with the CaM-tag was compared to the His$_6$-, GST-, and KSI-fusion expression systems. After induction by IPTG, the OD_{600} values for the latter systems are stagnant for at least 4 h, indicating that the expression of the toxic peptides inhibits culture growth during the mid-logarithmic phase. Although the KSI-fusion system is designed to direct the expressed proteins into inclusion bodies,[58] the KSI-tagged melittin and L-sel$_{318-372}$ constructs apparently still exert growth inhibiting activities. A SUMO-tag has been proposed previously for effective expression of many antimicrobial peptides;[59] however, SUMO-tag fusion constructs for some of our peptides such as melittin and L-sel$_{318-372}$ resulted in poor growth after IPTG induction (data not shown). By comparison, all the *E. coli* cells expressing CaM-fused peptides continue with an uninterrupted exponential growth phase to reach an $OD_{600} > 1.2$ after 4 h of induction. However, the final cell culture densities were slightly smaller compared to cultures expressing the CaM-tag unattached to a peptide (Figure S2). The overexpression of a series of CaM-peptide constructs was confirmed by SDS-PAGE (Figure S3). There was no detectable expression of L-sel$_{318-372}$ in the non-CaM fusion constructs as seen by SDS-PAGE.

During expression and purification, the calcium-occupancy of CaM may affect the strength of peptide binding and therefore, the efficiency of the CaM-fusion system. The binding of melittin to calcium-free CaM has been shown to greatly increase its affinity for calcium,[60] which would promote the population of tight peptide-Ca^{2+}-CaM complexes. Our attempt

DOI: 10.1021/jacs.6b06781
J. Am. Chem. Soc. 2016, 138, 11318−11326

to obtain in-cell NMR signals for some CaM-peptide fusion proteins resulted in very poorly resolved spectra which may be due to interactions with intracellular components and/or they may exist as a mixture of apo- and Ca^{2+}-bound forms, except that the $^{1}H,^{13}C$ HSQC signals of the methionine methyl-groups of *E. coli* cells expressing an ^{13}C-labeled CaM-fowlicidin-1 fusion could confirm the presence of the Ca^{2+}-bound form in the cytoplasm of *E. coli* (Figure S4). We also acquired a $^{1}H,^{15}N$ HSQC NMR spectrum of the diluted cell lysate of a culture expressing an ^{15}N-labeled CaM-fused peptide, and compared it to the same sample in either calcium-added or calcium-free conditions (Figure S5a). Despite the nonideal spectral quality due to interference from the contents of the lysate, the HSQC spectral overlays generally show that the spectrum for CaM-tritrpticin in the cell lysate matches more closely to the calcium-loaded spectrum than to that acquired in the presence of EDTA. This may suggest that the CaM-tritrpticin construct is also in the calcium-bound state in the bacterial cell, in contrast to a recent study with lysate NMR spectra showing that CaM alone expressed in *E. coli* is mainly in the apo-form.[61] Therefore, our observations are consistent with the enhanced Ca^{2+}-affinity seen for CaM-peptide complexes. It is known that the presence of target peptides can increase the Ca^{2+}-affinity of CaM by up to 2 orders of magnitude,[62] and this effect may be emphasized in the fusion proteins. Therefore, the population of Ca^{2+}-bound form of CaM-peptide fusion in the bacterial cell may vary depending on the fused peptide. However, in case the apo-form is dominant, apo-CaM can also interact with many targets,[63] and our SPR studies showed that most of the AMPs tested here can also associate with apo-CaM, albeit weakly (data not shown). Therefore, it is likely that fused CaM constructs can reduce the toxicity of the AMPs to some extent even for the apo-form of CaM. HSQC spectra were also acquired after the CaM-fused peptide was purified with a Ni^{2+}-column, and the results confirm the CaM-tritrpticin fusion remains in a calcium-saturated state (Figure S5b). If one were to be concerned about degradation during the purification of an AMP that is fused with CaM, a rational strategy would be to supplement all the purification buffers with $CaCl_2$.

Expression and Purification of CaM-Fused AMPs. Full scale purifications of several peptides were performed using the CaM-fusion construct. The peptides include melittin, tritrpticin, fowlicidin-1, and L-sel$_{318-372}$, and their constructs were all effectively expressed (Figure S3). CaM itself is a highly soluble protein,[38] which makes it ideal as a solubility tag for AMPs which are known for their high hydrophobicity. Indeed, the CaM-peptide constructs all remain in the supernatant portions of the cell lysates after high-speed centrifugation. This makes the purification of the protein constructs simpler and faster than the KSI and other inclusion-body-promoting tags, where high amounts of urea or guanidium-HCl are normally used for solubilization.[28,64,65]

As the CaM partner was engineered to possess an N-terminal $(His)_6$-tag, purification could be achieved directly with Ni^{2+}-column chromatography (Figure 5).

The amount of CaM-fusion protein construct eluted from this step varied from 25 to 60 mg per liter of culture. After an overnight dialysis step into digestion buffer, the purified proteins were subjected to TEV protease cleavage. TEV protease can be expressed and purified recombinantly,[52] which avoids the high cost of using commercially available proteases such as Factor X and enterokinase. The isolated

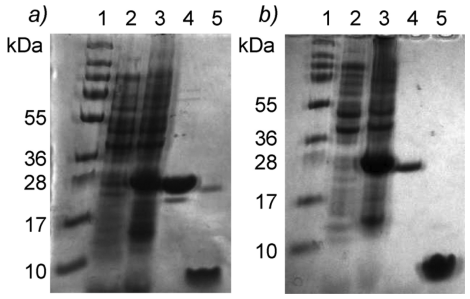


Figure 5. SDS-PAGE showing the expression and purification of (a) melittin and (b) tritrpticin. In both panels, lane 1 shows the molecular mass marker. Lanes 2 and 3 represent the *E. coli* cell lysate before and after IPTG induction, respectively. Lane 4 represents the peak fraction eluted from the Ni^{2+}-column. Lane 5 shows the final peptides purified by HPLC.

peptides were readily separated from CaM and TEV protease by passing through a C_{18} HPLC column (Figure 6).

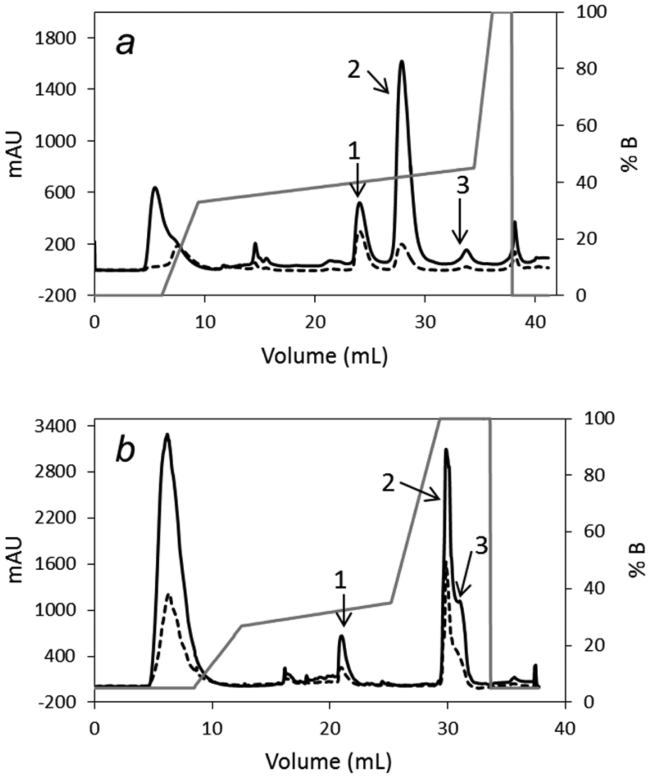


Figure 6. RP-HPLC chromatograms obtained for the purification of (a) melittin and (b) HBD-3 after TEV protease digestion. Indicated by the arrows are the purified peptides (1), cleaved CaM (2), and the remaining undigested CaM-peptide construct (3). Solid and dashed lines represent the absorbance at 220 and 280 nm, respectively. Gray line represents the acetonitrile (buffer B) gradient.

The yield for each purified AMP is listed in Table S1, and the purity was confirmed by HPLC (Figure S6). Most of the recombinant AMPs purified in this study retain a cloning artifact Gly-Thr addition at the N-terminus following TEV protease digestion. Recently, we have demonstrated that this artifact does not affect the antimicrobial activity of recombinant tritrpticin.[66] The efficiency of TEV protease can accommodate several substitutions at the P1′ site, especially with smaller residues;[67] therefore, this artifact does not necessarily need to

DOI: 10.1021/jacs.6b06781
J. Am. Chem. Soc. 2016, 138, 11318−11326

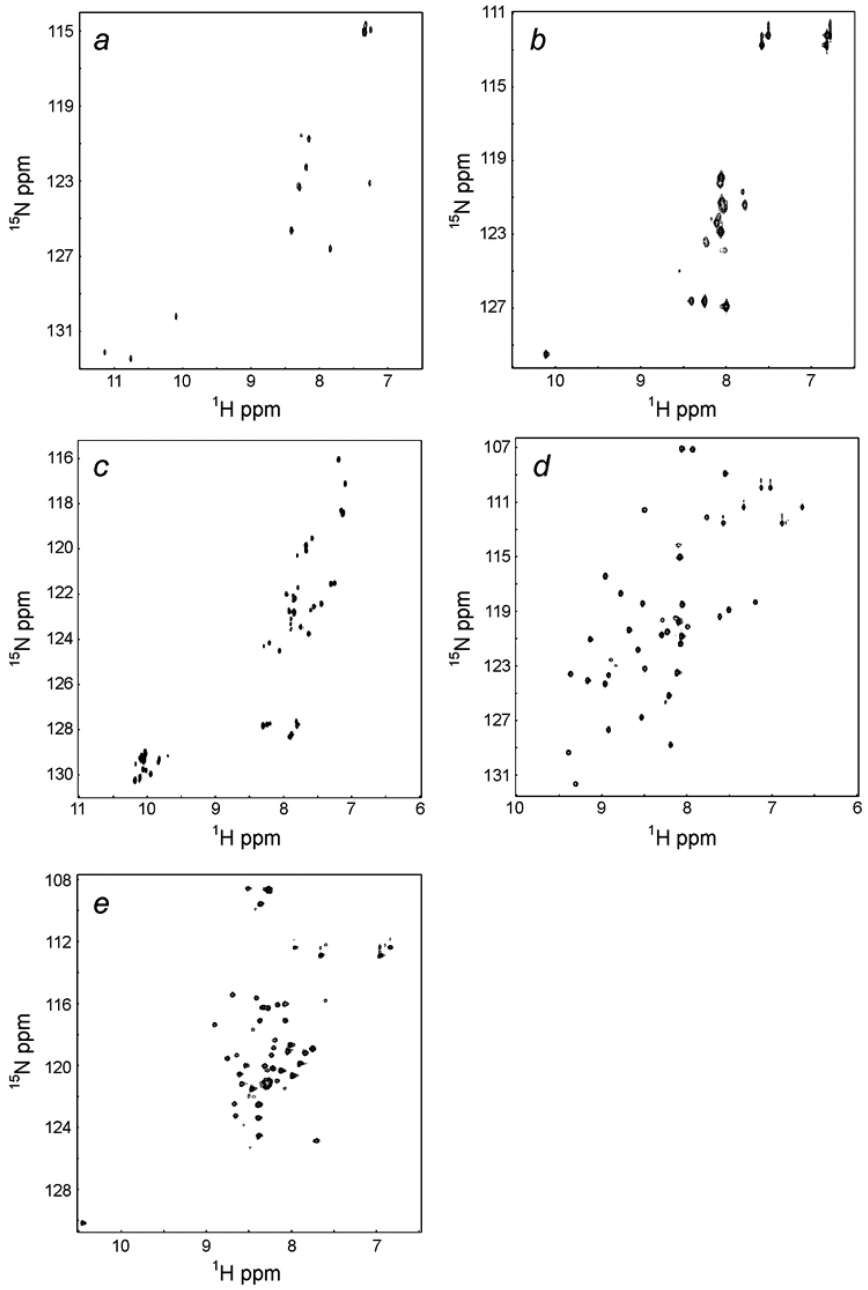


Figure 7. ^{1}H,^{15}N-HSQC spectra of ^{15}N-labeled (a) tritrpticin, (b) melittin, (c) indolicidin, (d) HBD3, and (e) L-sel$_{318-372}$. For further explanation see text.

be present for future CaM-peptide constructs. The entire purification procedure from cell lysis to HPLC takes 2 days, with a few more days needed for lyophilization of the collected peptide fractions.

Figure 7 shows the ^{1}H,^{15}N-HSQC NMR spectra of purified ^{15}N-labeled peptides in different environments. Figure 7a displays the HSQC spectrum of ^{15}N-labeled tritrpticin bound to DPC micelles. Tritrpticin is a 13-residue peptide containing three central tryptophan residues which exert a strong antimicrobial activity against both Gram-positive and Gram-negative bacteria.[42] Recently, we have demonstrated that this peptide may directly inhibit the DNA/RNA synthesis machinery in addition to causing membrane damage to kill bacteria.[68] The characteristic indole HN signals from the Trp residues are clearly visible at ∼10 ppm in the amide proton region of the NMR spectrum. In Figure 7b, ^{15}N-labeled melittin was dissolved in aqueous solution. The relatively broad signals are due to a tetramer to monomer transition that can occur under our experimental conditions for this peptide.[69] Figure 7c shows the spectrum obtained for ^{15}N-labeled indolicidin which was dissolved in aqueous solution. In the absence of a membranous environment, there are multiple sets of peaks present for this 13-residue peptide, three of which are Pro residues. The peptide is not folded and flexible in this state, and there may be signals coming from at least three different *cis*–*trans* isomers about the Pro residues. This result is consistent with previous ^{1}H NMR spectra recorded for this peptide.[70]

We have also used the CaM-fusion system to successfully purify a larger antimicrobial peptide, HBD-3, which presents the additional challenge of having a folded globular structure

DOI: 10.1021/jacs.6b06781
J. Am. Chem. Soc. 2016, 138, 11318–11326

with three intramolecular disulfide bonds. The procedure was amended to promote the correct folding of the peptide, and the CaM-HBD-3 construct was expressed in OrigamiB (DE3) *E. coli* cells; TEV digestion was carried out in a reduced/oxidized glutathione mixture instead of DTT in order to retain the disulfides while providing enough reducing power for TEV protease to work. The HPLC profile of the digest in Figure 6b shows one major peak for the cleaved peptide. Figure 7d displays the HSQC spectrum of ^{15}N-labeled HBD-3. The dispersed signals confirm the presence of a single well-folded conformation, and the HN chemical shift assignments are consistent with previous proton NMR assignments.[48] Thus, with the use of the CaM-tag, we could purify a correctly folded disulfide-containing AMP without requiring *in vitro* refolding procedures.[71]

In Figure 7e, the HSQC spectrum of the transmembrane domain of human L-selectin reconstituted into DHPC micelles indicates that it is folded in a typical helical conformation. L-selectin is a human cell adhesion receptor that is exclusively expressed in leukocytes and plays an important role in leukocyte-endothelial cell interaction.[72] The structure of L-selectin is composed of extracellular lectin and epidermal growth factor (EGF) domains, and a short cytoplasmic tail that is connected through a single transmembrane segment. Following cell activation, the extracellular domains are rapidly shed by a specific cleaving enzyme, ADAM17.[73] Interestingly, this event is controlled by CaM binding to the cytoplasmic tail of L-selectin in a calcium dependent manner.[74,75] Recently, we have proposed a structural model for how intracellular CaM may regulate the extracellular event.[34] However, there is no reported evidence that the structural change occurring in the cytoplasm is propagated to the extracellular domains through the plasma membrane. With the availability of this isotope-labeled transmembrane domain of L-selectin and the use of recently introduced nanodiscs,[19−21] we can investigate this mechanism further. The successful purification of this peptide shows that the CaM-fusion expression strategy can be extended beyond toxic AMPs and be useful for the expression and purification of transmembrane segments, which typically present challenges in terms of solubility and toxicity.[76,77] Many isolated transmembrane α-helices with their high content of exposed hydrophobic residues together with basic flanking residues on the cytosolic side of the membrane are likely to interact with and at least be partially covered by CaM as a fusion partner.

4. CONCLUSION

In this work, we have introduced a new CaM-fusion tag system as an expression vector to overcome the problems associated with the growth and expression inhibiting effects of producing antimicrobial and toxic transmembrane peptides in an *E. coli* host. This approach also counters the instability of AMPs during bacterial production, which has often been observed with other fusion-protein systems. Furthermore, the relatively small molecular size of CaM (16.7 kDa) would have less impact on the yield of the peptides compared to larger carrier proteins such as GST or maltose-binding protein. We were successful in obtaining fully isotope-labeled antimicrobial and anticancer peptides and acquired high-quality NMR spectra for these. In addition, the same strategy can be used for isotope-labeling of specific amino acids in the peptides, a strategy that is often used in solid state NMR studies.[78] Importantly, our approach extends beyond the production of AMPs to the transmembrane domain of membrane proteins. It is also possible to incorporate unnatural amino acids into various AMPs, such as tryptophan analogues[79] and fluorinated amino acids[66,80] using specialized media supplemented with these amino acids. Therefore, we can propose the CaM-fusion expression system as an effective and almost universal approach to produce various types of cationic amphipathic peptides that are normally toxic to *E. coli*. The availability of isotope-labeled material will allow us and other researchers to collect structural and dynamic NMR data for many AMPs as well as for selected transmembrane protein domains.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b06781.

Table S1 containing the sizes, pI values, and yields of AMPs, and Figures S1−S6 including NMR spectra, control expression of CaM-tag, SDS-PAGE showing expression of CaM-AMPs, in-cell-NMR spectrum of CaM-fowlicidin-1, RP-HPLC chromatograms, PCR primer lists, and M9 minimal media recipe (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*vogel@ucalgary.ca

Notes

The authors declare no competing financial interest.

†On leave from the Faculty of Advanced Life Science, Hokkaido University, Sapporo 060-0810, Japan.

■ ACKNOWLEDGMENTS

H.J.V. holds the Armstrong Chair in Molecular Cancer Research. R.G. received an Eyes-high postdoctoral fellowship from the University of Calgary.

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