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Lipid interactions of acylated tryptophan-methylated lactoferricin peptides by solid-state NMR‡

DENISE GREATHOUSE,* VITALY VOSTRIKOV, NICOLE MCCLELLAN, JUAN CHIPOLLINI, JACK LAY, ROHANA LIYANAGE, and TAYLOR LADD

Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, Arkansas 72701, USA

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Abstract: Lactoferricin (LfB) is a 25-residue innate immunity peptide released by pepsin from the *N*-terminal region of bovine lactoferrin. A smaller amidated peptide, LfB6 (RRWQWR-NH_2) retains antimicrobial activity and is thought to constitute the "antimicrobial active-site" (Tomita, *Acta Paediatr Jpn.* 1994; **36**: 585–91). Here we report on *N*-acylation of 1-Me-Trp^5-LfB6, Cn-RRWQ[1-Me-W]R-NH_2, where Cn is an acyl chain having n = 0, 2, 4, 6 or 12 carbons. Tryptophan 5 (Trp^5) was methylated to enhance membrane binding and to allow for selective deuteration at that position. Peptide/lipid interactions of Cn-RRWQ[1-Me-W]R-NH_2 (deuterated 1-Me-Trp^5 underlined), were monitored by solid state ^{31}P NMR and ^2H NMR. The samples consisted of macroscopically oriented bilayers of mixed neutral (dimyristoylphosphatidylcholine, DMPC) and anionic (dimyristoylphosphatidylglycerol, DMPG) lipids in a 3 : 1 ratio with Cn-RRWQ[1-Me-W]R-NH_2 peptides added at a 1 : 25 peptide to lipid ratio. ^2H-NMR spectra reveal that the acylated peptides are well aligned in DMPC:DMPG bilayers. The ^2H NMR quadrupolar splittings suggest that the 1-Me-Trp is located in a motionally restricted environment, indicating partial alignment at the membrane interface. ^{31}P-NMR spectra reveal that the lipids are predominantly in a bilayer configuration, with little perturbation by the peptides. Methylation alone, in C0-RRWQ[1-Me-W]R-NH_2, resulted in a 3–4 fold increase in antimicrobial activity against *E. coli*. *N*-acylation with a C12 fatty acid enhanced activity almost 90 fold. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: acylated antimicrobial peptide; arginine; lactoferricin; lipid bilayer; 1-methyl-tryptophan; solid-state NMR; tryptophan

INTRODUCTION

A dramatic increase in multidrug-resistant bacteria in recent years has led to intense searches for new antimicrobial compounds with different mechanisms of action. Antimicrobial peptides, components of the innate immune system of most organisms [1,2], are strong alternative antibiotic candidates [(3)]. These peptides are relatively short amphipathic molecules, consisting of fewer than 50 amino acids, with α-helical, β-sheet or loop conformations. Most have a net positive charge due to an excess of cationic amino acids such as Lys, Arg, and/or His, and over 50% hydrophobic amino acids [4]. Although many questions regarding their exact mechanism(s) of action remain unanswered, it is generally agreed that these peptides initially interact electrostatically with negative charges on bacterial membranes (lipopolysaccharide or lipoteichoic acid of gram-negative and gram-positive bacteria, respectively). While it is clear that the next steps involve some sort of membrane perturbation, the actual mechanism for killing the target cells is not known. Possibilities include the formation of transient pores, general membrane disruption, or, in some cases, membrane permeabilization and interaction with internal targets [2,5]. One class of antimicrobial peptides, rich in Trp and Arg, show high levels of antimicrobial activity, emphasizing the importance of these two amino acids for future drug design [6,7].

Lactoferricin B (LfB) is a 25-residue peptide with potent broad-spectrum antibiotic and antitumor activities that is released by pepsin from the *N*-terminal region of bovine lactoferrin [8]. In addition to numerous positively charged amino acids, a 15-amino acid derivative of LfB, FKC**RRW**Q**WR**MKKLGA (residues 17–31 of mature bovine lactoferrin), contains two tryptophans, flanked by three arginines (in bold). The two tryptophans are absolutely required for antimicrobial activity [9]. Fluorescence measurements have suggested that the Trp indole rings reside in the interfacial region of micelles and unilamellar vesicles [8]. An amidated hexapeptide, (LfB6: RRWQWR-NH_2), retains significant activity, indicating that it may constitute a core active

Abbreviations: CSA, chemical shift anisotropy; DMPC, 1,2-dimyristoylphosphatidylcholine; DMPG, 1,2-dimyristoylphosphatidylglycerol; LfB, lactoferricin bovine; LfB6, lactoferricin bovine hexapeptide (RRWQWR-NH_2); MBC, minimal bactericidal concentration; 1-Me-Trp, 1-methyltryptophan; MHB, Mueller Hinton broth; MIC, minimum inhibitory concentration; QCC, quadrupolar coupling constant; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TIPS, triisopropylsilane.

* Correspondence to: Denise Greathouse, Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, Arkansas 72701, USA; e-mail: dgreatho@uark.edu

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region or perhaps even the 'antimicrobial active site' [10]. In aqueous solutions, LfB6 is unstructured; however, upon binding to SDS micelles it adopts a well-defined amphipathic conformation with Trp indole rings more deeply buried in the micelle than the Arg side chains, which are located at the surface [11]. Studies of similar Arg- and Trp-rich peptides revealed that three Arg and two Trp residues along with an amidated *C*-terminal are the minimum requirements for high antimicrobial activity and low toxicity [6,7,12,13].

Recent studies have indicated that lipid–peptide hybrid compounds show increased antimicrobial activity, pathogen selectivity [14–16], as well as resistance to proteases [15,17]. Several groups have attached acyl chains of varying lengths to lactoferricin peptides, at their amino [18–22] or carboxy ends [20,23]. Non-natural amino acids also show promise for enhancing antimicrobial activity [24], increasing protease resistance [25–27], and decreasing the immunostimulatory properties of antimicrobial peptides [28]. Methylation of the Trp indole ring could promote a deeper binding at the membrane interface [29] and thereby, may also increase the antimicrobial activity of Trp-rich peptides.

In recent years, solid-state NMR spectroscopy has gained popularity as a convenient and nonperturbing tool to monitor reciprocal interactions of membrane-bound peptides. The 100% natural abundance, high gyromagnetic ratio, and chemical shift anisotropy (CSA), which is sensitive to the polymorphic phase (state) and headgroup conformation of phospholipids [30], make the ^{31}P isotope an ideal probe for membrane lipids [31,32]. Concurrently, peptides labeled at specific positions with ^{2}H [33,34], ^{15}N [35,36] or ^{19}F [37] can provide details about the structure and dynamics of peptides when bound to membranes. These methods, when used together, are able to provide complementary information that may help elucidate the mechanism by which antimicrobial peptides exert their effects on membranes.

Here, we report on the *N*-acylation of 1-Me-Trp^5-LfB6 and Cn-RRWQ[1-Me-W]R-NH_2, where Cn is an acyl chain having $n = 0$, 2, 4, 6 or 12 carbons. Trp^5 was methylated to enhance membrane binding [29] and to allow selective deuteration at that position (deuterated residue underlined) [38]. Peptide/lipid interactions of Cn-RRWQ[1-Me-W]R-NH_2, in macroscopically oriented bilayers of mixed neutral and anionic lipids (DMPC : DMPG, 3 : 1) were monitored by solid-state ^{31}P NMR and ^{2}H NMR. The ^{2}H-NMR spectra demonstrate that acylated peptides align well and are located in a motionally restricted environment. The ^{31}P NMR spectra reveal that the lipids are predominantly in a bilayer phase with little evidence of perturbation to the phospholipid headgroup by the peptides. Antimicrobial assays against *Escherichia coli* (*E. coli*) demonstrated that methylation alone, in C0-RRWQ[1-Me-W]R-NH_2, was sufficient to produce a 3 to 4-fold increase in activity. *N*-acylation of the methylated LfB6 with $n = 2$, 4, or 6 carbons gave similar activity compared with methylation alone, whereas, acylation with a C12 fatty acid, in C12-RRWQ[1-Me-W]R-NH_2, enhanced activity almost 90 times.

MATERIALS AND METHODS

Peptides

L-1-methyl-tryptophan (Sigma-Aldrich, St. Louis, MO) was manually derivatized with an *N*-terminal Fmoc protecting group [39] and recrystallized from ethyl acetate/hexane (95/5, v/v). Peptides were synthesized by solid-phase Fmoc methods on an Applied Biosystems (Foster City, CA) 433A peptide synthesizer using modified FastMoc chemistry. Specifically the first amino acid, Fmoc-Arg(Pbf)-OH (Pbf: 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl), was loaded on Rink Amide resin (NovaBiochem, San Diego, CA) by double coupling with 10-fold excess amino acid and 6-h coupling times. The remaining amino acids (Fmoc-Trp(Boc)-OH, Fmoc-1-Me-Trp-OH, and Fmoc-Gln(Trt)-OH (*N*-α-Fmoc-*N*-γ-trityl-L-glutamine) were added using extended deprotection (>30 min) and 90-min coupling times. The butanoyl (C4), hexanoyl (C6), and dodecanoyl (C12) peptides were completed by coupling with butanoic, hexanoic acid, or dodecanoic acid, respectively, while attached to the resin. The acetylated (C2) peptide was completed by capping with acetic anhydride. The peptides were simultaneously cleaved from the resin, deprotected, and the 1-Me-Trp was selectively deuterated *in situ* by treatment for 3 h while shaking with a mixture of 85% TFA-d_1, 5% TIPS, 5% phenol, and 5% D_2O at 22 °C [38]. In some cases, D_2O was used instead of H_2O to prevent back-exchange of the methylindole ring. (The amount of D_2O used is standard for peptide cleavage reactions and is insufficient to quench the deuteration reaction.) The peptides were separated from the resin by filtration, rinsed once with TFA-d_1, and the filtrate volume was reduced approximately 4-fold under N_2. After precipitation with cold *tert*-butylmethyl ether/*n*-hexane (50/50, v/v) and centrifugation at 4 °C (three times), the peptides were lyophilized to white powder from acetonitrile/water (50/50, v/v) under high vacuum. Peptides were characterized by reversed-phase HPLC and MS. Figure 1 shows representative chromatograms of C4 and C12 acylated peptides. Only one major peak was observed at 13.9 and 16.5 min, for C4 and C12 peptides, respectively; therefore, peptides were used without further purification. Furthermore, mass spectra (Figure 2) showed no evidence of (n-1) peptides missing a single residue.

Mass Spectrometry

MS was used to determine the number and location of deuterium atoms incorporated into the synthetic peptides (Figure 2). MALDI mass spectra were obtained using a Bruker Reflex III MS. ESI mass spectra and MS/MS (product ion spectra from the protonated molecule) were obtained using a Bruker Esquire ESI LC/MS/MS. MALDI, ESI, MS, and MS/MS conditions were standard for these instruments. A representative MALDI spectrum of C6-RRWQ[1-Me-W]R-NH_2 is shown in Figure 2(a), revealing a molecular mass consistent with incorporation of about four deuterium atoms. An ESI

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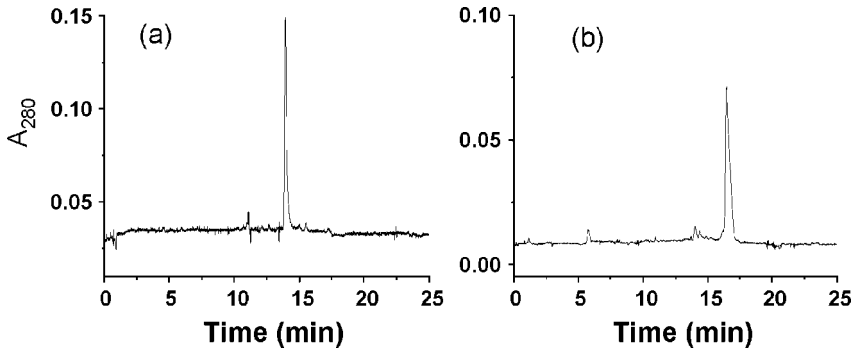

Figure 1 RP-HPLC chromatograms of (a) C4-RRWQ[1-Me-W]R-NH_2 and (b) C12-RRWQ[1-Me-W]R-NH_2 showing 1 major peak for each peptide. (Column: Zorbax SB-C8, 4.6 × 50 mm, 3.5 μm (Agilent Technologies, Wilmington, DE); Gradient: 10% MeOH (including 0.1% TFA) for 2 min, 10 to 60% over 8 min, followed by a hold at 60% for 5 min; 1 ml/min; 22 °C).

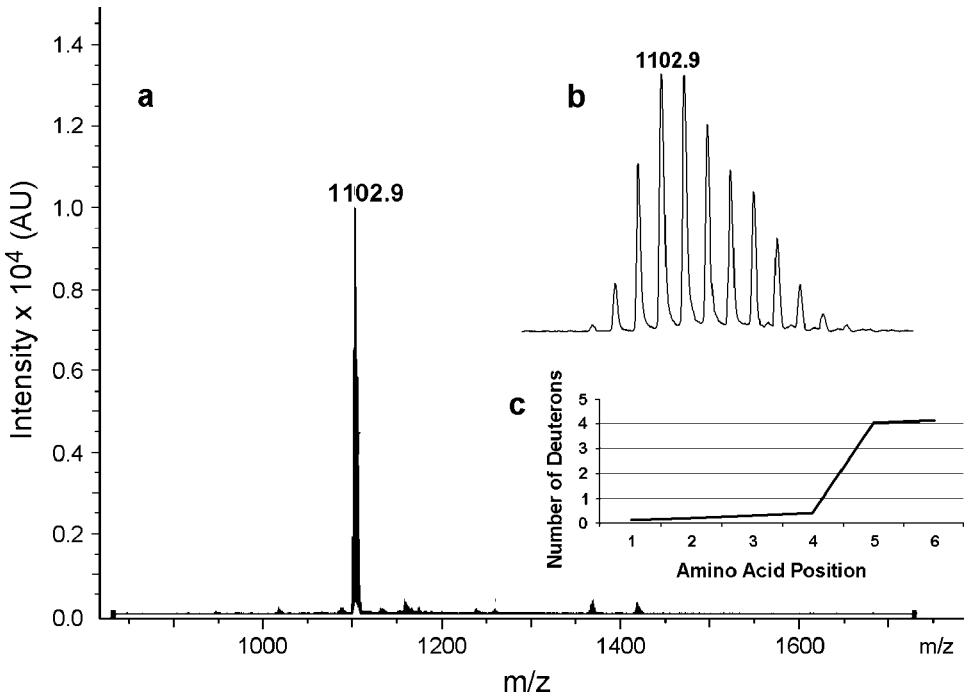

Figure 2 MS results for C6-RRWQ[1-Me-W]R-NH_2: (a) MALDI TOF mass spectrum, (b) expansion of the protonated molecule region showing four Dalton mass shifts compared to the expected profile (not shown), and (c) the mass shifts (observed compared to expected for undeuterated peptide) observed in 'b' and 'y' sequence fragment ions from the protonated molecule obtained in ESI MS/MS experiments.

spectrum (not shown), which encompasses the full mass range from 0 to 3000 m/z also revealed no evidence of lower molecular weight contaminants. The inset, Figure 2(b), is an expansion of the protonated molecule region showing a mass shift (1102.9) compared to the expected profile of the nondeuterated MH^+ (1099.3). Peptide sequencing by ESI tandem MS then allowed the deuterium incorporation to be localized to individual amino acids based on the mass values for the 'b' and 'y' ions obtained using low-energy collisions. The observed shifts in the isotope profiles for these fragments, when compared to the theoretical isotope profiles, as shown in Figure 2(c), demonstrate fractional deuteration at random locations that sum to about half a deuteron per peptide, together with about four deuterium atoms incorporated as expected at 1-Me-Trp^5.

ANTIMICROBIAL ASSAYS

The antimicrobial activity against *E. coli* (ATCC 25 922) was assayed for native and deuterated acylated peptides having $n = 0$, 2, 4, 6, or 12. The MIC and minimal bactericidal concentration (MBC) of each peptide were determined using standard microdilution broth assay methods [40], adapted for cationic peptides by Hancock [41]. Briefly, 500 μl of overnight cultures in Mueller Hinton broth (MHB) was diluted in 25 ml fresh MHB, incubated at 37 °C to exponential phase (optical density at 600 nm of 0.6), and then diluted 1×10^{-5} in fresh MHB. Serial dilutions (200, 100, 50, 25, 12.5, 6.25, 3.125, 1.6 μg/ml) of each peptide were made in 0.2%

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bovine serum albumin–0.01% acetic acid solution. Each well of a 96-well polypropylene microtiter plate was inoculated with a total volume of 100 µl (approximately 1×10^8 colony forming units/ml). The MIC was taken as the lowest peptide concentration at which growth was inhibited after 24 h of incubation at 37 °C as determined by measuring optical density at 600 nm. The MBC was confirmed when no bacterial colonies were observed after 100 µl from the 96-well plate was spread onto an MHB agar plate and incubated overnight at 37 °C. Each peptide was assayed at least twice.

NMR Spectroscopy

Samples for solid-state NMR spectroscopy, consisting of 4 µmol peptide and 100 µmol lipid hydrated to at least 40% with deuterium-depleted water, were prepared on the basis of the stacked glass plate procedure outlined by van der Wel [33]. Samples were prepared with 4 mol% peptide to ensure adequate ^{2}H NMR signals, and were equilibrated at 40 °C for at least 48 h. Some samples contained only DMPC, others a mixture of DMPC : DMPG (3 : 1). Once optical clarity of the glass plate samples was achieved, NMR measurements were performed at 46.0 MHz (^{2}H) and 121.4 MHz (^{31}P) using Bruker Avance 300 spectrometers. The experimental temperature and hydration levels were maintained to ensure that the DMPC and DMPG lipids were above their respective gel-to-liquid crystalline transition temperatures of about 23 °C [42]. To avoid artifacts associated with lipid hydrolysis, which was observed in some samples after 4–6 weeks, NMR spectra were acquired within 10 days after sample preparation.

The ^{2}H measurements involved 1–2 million acquisitions and were done using a quadrupolar echo pulse sequence with full-phase cycling [43], an echo delay of 100–125 µs, a pulse length of 3 µs, and a 60-ms interpulse delay time. Spectra were recorded with the lipid bilayer normal aligned either parallel to the magnetic field, $\beta = 0°$; or perpendicular, $\beta = 90°$. Line-broadening of 500 Hz was applied to all spectra. Quadrupolar splittings ($\Delta\nu_q$) were measured as the distances between corresponding peak maxima.

The ^{31}P spectra were acquired using the Bruker 'zgpg' pulse program with 256 scans, a 6-µs 90° pulse, and an interpulse delay time of 5 s. Measurements were carried out in a Doty 8-mm wideline probe (Doty Scientific Inc., Columbia, SC) with broadband ^{1}H decoupling. The chemical shift was referenced externally to 85% phosphoric acid at 0 ppm.

RESULTS AND DISCUSSION

Antimicrobial Assays

The peptides were tested for activity against *E. coli* in 96-well plates using a standard microdilution broth assay adapted for antimicrobial peptides by Hancock, *et al.* [41]. The lowest peptide concentration found to inhibit bacterial growth after incubation at 37 °C for 24 and 48 h, as determined by measuring optical density at 600 nm, was taken to be the MIC. The MBC for each of the peptides was confirmed when no growth was observed after 100 µl from one of the wells was spread onto an MHB agar plate and incubated overnight at 37 °C. MIC and MBC values are reported in Table 1.

As shown in Figure 3 (shaded bars) methylation alone of C0-RRWQ[1-Me-W]R-NH_2 inhibited growth of *E. coli* approximately 3-fold compared to native LfB6. Additional acylation of 1-Me-Trp LfB6 with $n = 2$, 4, or 6 resulted in no further inhibition of growth. Acylation with $n = 12$, however, inhibited bacterial growth approximately 22-fold compared to native LfB6. Also shown in Figure 3 (clear bars) are the MBC results. Bactericidal activity increased almost 4-fold in the methylated only peptide, C0-RRWQ[1-Me-W]R-NH_2, compared to native LfB6. The addition of an acyl chain up to $n = 6$ did not significantly improve the bactericidal activity of the peptides compared with C0-RRWQ[1-Me-W]R-NH_2. The most dramatic results, however, were observed for C12-RRWQ[1-Me-W]R-NH_2, where the bactericidal activity was increased by almost 90-fold compared with native LfB6.

These results indicate that the combination of Trp-methylation and acylation contribute to the antimicrobial activity against *E. coli*. Our findings further support those of others that acylation [23], particularly with the hydrophobic C12, is an effective means to enhance antimicrobial activity of membrane-active peptides [14,20,23,44,45]. The findings are especially significant in light of the fact that drugs with increased bactericidal effects have been reported to reduce the development of bacterial resistance mechanisms [46]. It remains to be tested whether *N*-acylation with C12 alone would be sufficient to produce the same results.

Solid-State NMR Spectroscopy

Solid-state NMR spectroscopy in oriented lipid bilayers was performed to examine the effects of attaching acyl

Table 1 Activity (MIC and MBC) of LfB6 peptides against *E. coli* (ATCC 25 922)

LfB Peptide	MIC µg/ml	MBC µg/ml
Native	140	550
C0	50	150
C2	50	150
C4	50	150
C6	50	100
C12	6.25	6.25

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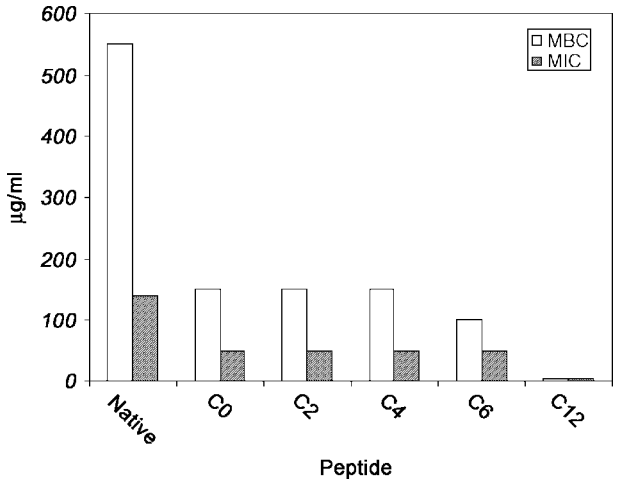

Figure 3 MIC (shaded bars) and MBC (clear bars) (µg/ml) for native LfB6 (RRWQWR-NH_2), and 1-Me-Trp peptides C0- through C12-RRWQ[1-Me-W]R-NH_2 against *E. coli* (ATCC 25 922).

groups to the amino terminal of the 'antimicrobial active site' of lactoferricin. Incorporation of 1-Me-Trp allowed for selective deuteration [38,47], thus yielding Cn-RRWQ[1-Me-W]R-NH_2 (deuterated residue underlined). A 3 : 1 mixture of DMPC : DMPG was used to approximate the surface charge of bacterial cell membranes [48].

^{2}H NMR

Initially, ^{2}H NMR was used to determine whether the acylated LfB peptides would align with respect to oriented bilayers of neutral or mixtures of neutral and anionic lipids. To examine this, Cn-RRWQ[1-Me-W]R-NH_2 peptides were mixed with DMPC : DMPG (3 : 1) and hydrated with approximately 40% (w/w) deuterium-depleted water. Figure 4 shows the ^{2}H NMR spectra of C6-RRWQ[1-Me-W]R-NH_2 mixed with hydrated lipids that are oriented at $\beta = 0^\circ$ (a) or $\beta = 90^\circ$ (b). A pair of symmetrical peaks is expected for each deuteron incorporated into a peptide. For perdeuterated Trp, four distinct pairs of peaks (doublets) are expected for deuterons attached to carbons C-2, C-4/7, C-5, and C-6. Owing to the indole ring geometry, deuterons attached to carbons 4 and 7 result in virtually identical quadrupolar splittings, although they may appear 'twinned' due to a slight deviation from a 180° relative orientation [49]. During *in situ* deuteration of Fmoc-Trp at 4 °C, favorable resonance intermediates in which the positive charge is shared with the indole nitrogen, result in rapid, selective isotope exchange at ring positions 2 and 5 [34]. In the case of *in situ* deuteration of peptides containing 1-Me-Trp, exchange is enhanced at positions 2 and 5 due to the electron donating effects of the methyl group [34]. For our peptides, which were simultaneously cleaved and exchanged at 22 °C, we find that the electron-withdrawing Boc-protecting group on the indole nitrogen of Trp^3 inhibits isotope exchange at the Boc-Trp^3 ring, while the exchange is enhanced on the Me-Trp^5 ring. For peptides exchanged during cleavage at 22 °C, therefore, up to four deuterons can be observed per methylindole ring. Additional signals probably emerge from (slower) exchange positions 4, 6, and 7 on the methylindole ring and contribute additional minor peaks to the spectra [34,50]. The isotropic signal observed in all samples could be due to the presence of residual ^{2}H-containing water or traces of TFA molecules in the samples, or it could be a splitting from one of the deuterons on the methylindole ring with a value of 0 kHz. Further experiments will be required to assign the peaks in the spectra.

The frequency difference between the two members of a given pair of peaks is the quadrupolar splitting, defined by the equation:

$$\Delta\nu_q = \frac{3}{2} S_{zz} \frac{e^2 qQ}{h} \left(\frac{1}{2}[3cos^2\theta - 1]\right)\left(\frac{1}{2}[3cos^2\beta - 1]\right) \quad (1)$$

where $\Delta\nu_q$ is the quadrupolar splitting magnitude, which is measured experimentally; e^2qQ/h is the quadrupolar coupling constant, ~180 kHz for aromatic deuterons [51], and β is the angle between the membrane normal and the magnetic field, either 0° or 90°, which is defined by the sample orientation. The key variable θ represents the angle between a particular ring C–D bond and the membrane normal. The reduction by half of the quadrupolar splittings ($\Delta\nu_q$) when $\beta = 90^\circ$ confirms that the peptide is indeed aligned and rotating rapidly with respect to the membrane normal. Figure 5(a) shows the ^{2}H NMR spectra of C0-RRWQ[1-Me-W]R-NH_2 in DMPC alone oriented at $\beta = 0^\circ$. Also shown in Figure 5 are samples prepared

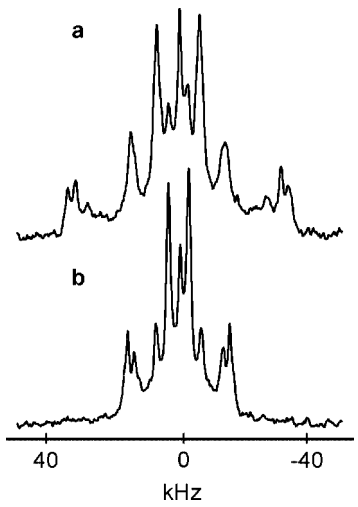

Figure 4 The ^{2}H NMR spectra of labeled 1-Me-Trp in C6-RRWQ[1-Me-W]R-NH_2 in hydrated DMPC : DMPG (3 : 1) at (a) $\beta = 0^\circ$ and (b) $\beta = 90^\circ$; 50 °C.

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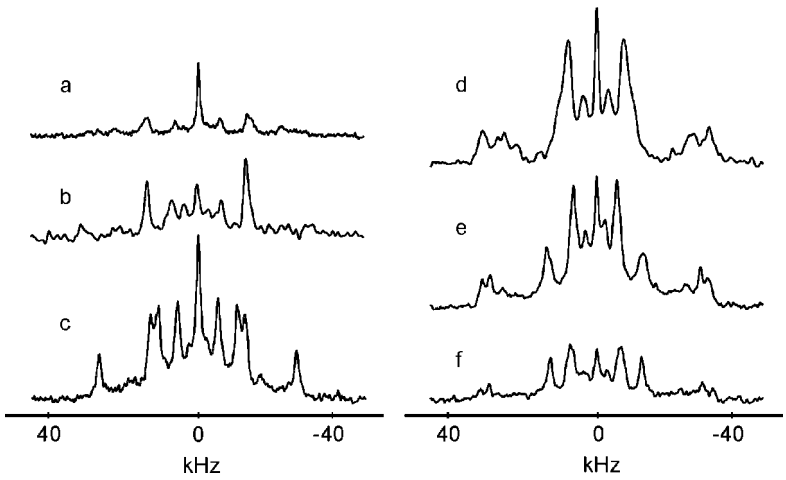

Figure 5 The ^{2}H NMR spectra of labeled 1-Me-Trp in C0-RRWQ[1-Me-W]R-NH_2 in hydrated (a) DMPC, and (b) DMPC : DMPG (3 : 1). Also shown are acylated RRWQ[1-Me-W]R-NH_2 peptides in DMPC : DMPG (3 : 1): (c) C2; (d) C4; (e) C6; and (f) C12. All spectra are at $\beta = 0^\circ$ and 50 °C; peptide/lipid ratio 1 : 25.

in DMPC : DMPG with C0-, C2-, C4-, C6-, and C12-RRWQ[1-Me-W]R-NH_2, oriented at $\beta = 0^\circ$. Quadrupolar splittings ($\Delta\nu_q$) for all spectra at $\beta = 90^\circ$ (not shown) give values that are very nearly half compared to those at $\beta = 0^\circ$, confirming that the peptides are aligned and rotating rapidly with respect to the membrane normal. Acylation, particularly with C2, C4, and C6, produced spectra with higher signal intensities and peak resolution compared with nonacylated C0-RRWQ[1-Me-W]R-NH_2 in DMPC or DMPC : DMPG. The presence of a negatively charged lipid, DMPG, was also required to produce a strong signal, as samples prepared with DMPC alone either produced weak or no signals (for example, Figure 5(a)), suggesting weaker peptide alignment, perhaps due to weaker peptides association with the membranes that lack the negatively charged DMPG. Table 2 lists the quadrupolar splittings ($\Delta\nu_q$) for deuterated 1-Me-Trp in the various acylated LfB6 peptides.

Since local and global motions tend to narrow the ^{2}H NMR spectra, the spectral width is a qualitative indicator of ring motion for deuterated Trp or 1-Me-Trp. The maximum quadrupolar splittings of 1-Me-Trp in Cn-RRWQ[1-Me-W]R-NH_2 peptides, 60–70 kHz (DMPC : DMPG; $\beta = 0^\circ$; 50 °C), are slightly higher than those observed for free methylindole in oriented DMPC bilayers (60 kHz at 30 °C and 45 kHz at 50 °C) [29]. We note that even free methylindole exhibits partial alignment (not isotropic) in oriented lipid bilayers [29] and represents a 'lower bound' for the expected alignment of indole rings in peptides. The observed maximal $\Delta\nu_q$ values (Table 2) suggest that the 1-Me-Trp of LfB6 is marginally more aligned than the free methylindole, in a somewhat motionally restricted environment, most likely at the interface of the bilayer. Remarkably, the maximum observed quadrupolar splittings are quite different for Trp indole rings at the two ends of a membrane-spanning α-helix [49]. Not surprisingly, the implied alignment and motion of 1-Me-Trp^5 of LfB6, regardless of acylation, are comparable to the properties of the more mobile Trps in membrane-spanning α-helices.

Table 2 ^{2}H-quadrupolar splittings ($\Delta\nu_q$) for labeled MeTrp groups in acylated LfB6 peptides at $\beta = 0^\circ$; all at 50 °C except as noted[a]

Peptide	$\Delta\nu_q$ (kHz), DMPC : DMPG (3 : 1)
[a]C0	[a]68, 46, 30, 15, 7, 0
C2	60, 28, 24, 12, 6, 0
C4	69, 57, 47, 17, 8, 0
C6	68, 63, 29, 13, 6, 0
C12	70, 64, 27, 16, 7, 0

[a] Sample measured at 40 and 60 °C.

^{31}P NMR

The effect on the lipids in the presence of acylated LfB peptides was investigated using ^{31}P NMR spectroscopy. When the samples are oriented at $\beta = 0^\circ$ or $\beta = 90^\circ$, with the bilayer normal perpendicular or parallel to the magnetic field (H_0), single peaks are expected at ~30 ppm or −17 ppm corresponding to the 0° or 90° edge of the ^{31}P CSA powder pattern, if no orientational disorder is present [33,52]. Disorder induced in the lipids by the peptide will result in shifts away from these positions [53]. Spectra of oriented hydrated samples of DMPC and DMPC : DMPG (3 : 1) are shown in Figure 6(a) and (b), respectively. A single predominant peak at approximately 26 ppm at the $\beta = 0^\circ$ orientation confirms that both samples are predominantly bilayers. A small amount of nonoriented lipid is also evident from the peak at −17 ppm in both samples.

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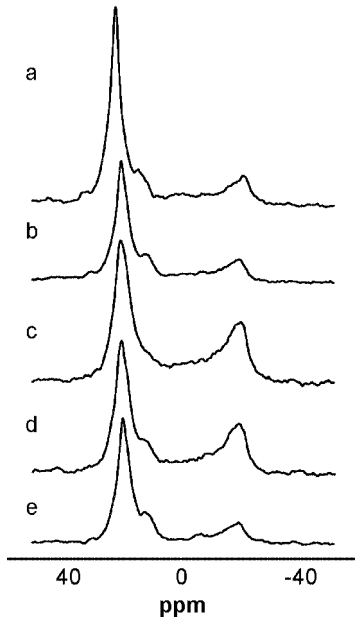

Figure 6 The ^{31}P NMR spectra of oriented hydrated (a) DMPC; (b) DMPC : DMPG (3 : 1); and (c) C0-RRWQMeWR-NH_2; (d) C6-RRWQMeWR-NH_2; and (e) C12-RRWQMeWR-NH_2 in hydrated DMPC : DMPG (3 : 1). All spectra are at $\beta = 0°$ and 50 °C; peptide/lipid ratio 1 : 25).

Spectra of hydrated oriented samples of the mixed DMPC : DMPG lipids with C0-RRWQ[1-Me-W]R-NH_2 (Figure 6(c)), C6-RRWQ[1-Me-W]R-NH_2 (Figure 6(d)), and C12-RRWQ[1-Me-W]R-NH_2 (Figure 6(e)) remain essentially unchanged. The spectra in Figure 6 suggest little variation with the length of the acyl chain on the peptide, as well as little observable perturbation to the lipid headgroups, even at 1 : 25 peptide-to-lipid ratio.

CONCLUSIONS

The ^{2}H-quadrupolar splittings observed for the 1-Me-Trp^5 in LfB6 peptides suggest that the indole ring resides in a motionally restricted environment, most likely at the membrane/water interface, and exhibits an extent of motion similar to tryptophans near one end of a membrane-spanning α-helix. Our results further support the concept of acylation, particularly for the longer C12 chain, as an effective means to enhance the activity of antimicrobial peptides. The incorporation of the non-natural amino acid 1-Me-Trp also shows promise as a means to increase the antimicrobial activity of Trp-rich peptides, as well as to probe the mechanisms by which these peptides exert their effects at the membrane.

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