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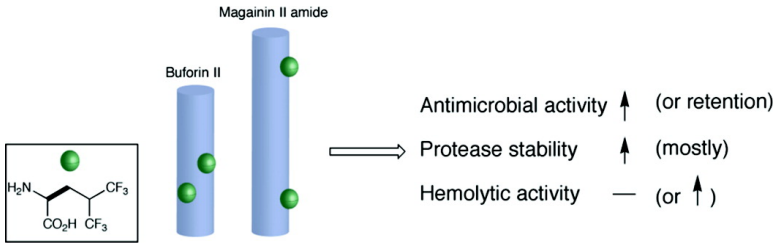

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Antimicrobial Activity and Protease Stability of Peptides Containing Fluorinated Amino Acids

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Abstract: Selective fluorination of peptides results in increased chemical and thermal stability with simultaneously enhanced hydrophobicity. We demonstrate here that fluorinated derivatives of two host defense antimicrobial peptides, buforin and magainin, display moderately better protease stability while retaining, or exhibiting significantly increased bacteriostatic activity. Four fluorinated analogues in the buforin and two in the magainin series were prepared and analyzed for (1) their ability to resist hydrolytic cleavage by trypsin; (2) their antimicrobial activity against both Gram-positive and Gram-negative bacterial strains; and (3) their hemolytic activity. All but one fluorinated peptide (**M2F5**) showed retention, or significant enhancement, of antimicrobial activity. The peptides also showed modest increases in protease resistance, relative to the parent peptides. Only one of the six fluorinated peptides (**BII1F2**) was degraded by trypsin at a slightly faster rate than the parent peptide. Hemolytic activity of peptides in the buforin series was essentially null, while fluorinated magainin analogues displayed an increase in hemolysis compared to the parent peptides. These results suggest that fluorination may be an effective strategy to increase the stability of biologically active peptides where proteolytic degradation limits therapeutic value.

Introduction

The emergence of bacterial resistance to common antibiotics poses a serious threat to human health[1] and has rekindled interest in antimicrobial peptides.[2,3] Both plants and animals have an arsenal of short peptides with diverse structures that are part of the innate immune system and are deployed against microbial pathogens. The common distinguishing characteristic among these peptides is their ability to form facially amphipathic conformations, segregating cationic and hydrophobic side chains.[2,4] The classification of these peptides has usually been on the basis of secondary structure. Both α-helical (magainins and cecropins) and β-sheet (bactenecins and defensins) secondary structure elements are well represented among the 500 plus members that have been hitherto identified.[2,4−6] Most eukaryotes express a combination of such peptides from many different classes within tissues that provide the first line of defense against invading microbes. The architectural details reveal the mechanism of action—positive charges help the peptides seek out negatively charged bacterial membranes, and the interaction of the hydrophobic side chains with the acyl chain region of lipid bilayers eventually leads to membrane rupture or translocation of peptides.[5,7,8] Because of the broad spectrum activity and ancient lineage of these peptides, it has been suggested that bacterial resistance may be completely thwarted or slowed down enough to offer a reasonably long therapeutic lifetime for suitable candidates.[2,9,10]

Strategies to modulate antimicrobial activity of host defense peptides have relied mainly on substitution at single (or multiple) sites by 1 of the other 19 natural amino acids. This approach has resulted in several improved variants, most notably the [Ala[8,13,18]]-Magainin II amide (**M2**, Figure 1).[11,12] On the other hand, general principles gleaned from the study of natural peptides have been utilized in the design of antimicrobial peptides and polymers using unnatural building blocks. Several of these constructs based on β-peptides,[13−17] D,L-α-peptides,[18] and arylamide polymers[19,20] show impressive bactericidal activity. We[21,22] and others[23−25] have recently described peptide

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assemblies that incorporate highly fluorinated residues that have superior thermal and chemical stability. The increased hydrophobicity of fluorinated amino acids fuels the formation of stable secondary and quaternary protein structures in aqueous solutions if the nonpolar fluorinated surfaces can be segregated away from water. Indeed, appropriately designed fluorinated peptides show a higher affinity for membranes as in the case of cell lytic melittin[26] and can also direct discrete oligomer formation in biological membranes.[27,28] We envisioned that increased membrane affinity and greater structural stability could yield peptide variants that have increased potency and stability toward proteolytic enzymes than known antimicrobials.

Experimental Section

Materials. Boc-Lys(2-Cl-Z)-Merrifield resin, 4-methylbenzhydrylamine (MBHA) resin, Boc-L-amino acids, and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Novabiochem. Dichloromethane (ACS grade, Fisher), dimethylformamide (sequencing grade, Fisher), *N*,*N*-diisopropylethylamine (biotech grade, Aldrich), trifluoroacetic acid (New Jersey Halocarbon), anisole (anhydrous, Aldrich), trifluoroethanol (TFE, 99%, Acros), and acetonitrile (Optima grade, Fisher) were used without further purification. Hydrogen fluoride was purchased from Matheson Gas. Melittin (from honey bee venom), trypsin (from bovine pancreas), and benzoyl arginine ethyl ester (BAEE) were purchased from Sigma. Fresh human red blood cells (type B) were procured from Research Blood Components L.L.C., Boston, MA. Chemical synthesis of hexafluoroleucine was carried out according to a previously disclosed procedure with slight modifications.[29] Solvents for reversed-phase high-performance liquid chromatography (RP-HPLC) had the following compositions: Solvent A, H_2O/CH_3CN/TFA (99/1/0.1); Solvent B, CH_3CN/H_2O/TFA (90/10/0.07).

Peptide Synthesis, Purification, and Characterization. Peptides were synthesized manually using the *in situ* neutralization protocol for *t*-Boc chemistry[30] on a 0.075 mmol scale. Boc-Lys(2-Cl-Z)-Merrifield and MBHA resins were used for buforin and magainin peptides. The dinitrophenyl protecting group on histidine was removed using a 20-fold molar excess of thiophenol. Peptides were cleaved from resin by treatment with HF/anisole (90:10) at 0 °C for 2 h and then precipitated with cold Et_2O. Crude peptides were purified by RP-HPLC [Vydac C_{18}, 10 μ, 10 mm × 250 mm]. The purities of peptides were judged to be greater than 95% by analytical RP-HPLC [Vydac C_{18}, 5 μ, 4 mm × 250 mm]. The molar masses of peptides were determined by MALDI-TOF MS. Peptide concentrations were determined by quantitative amino acid analysis.

MALDI-TOF MS Characterization: BII1 *m*/*z* calcd (M) 2432.4, obsd 2434.9 (M + H^+); **BII1F2** *m*/*z* calcd (M) 2649.3, obsd 2650.7 (M + H^+); **BII5** *m*/*z* calcd (M) 2002.2, obsd 2003.5 (M + H^+); **BII5F2** *m*/*z* calcd (M) 2218.1, obsd 2218.9 (M + H^+); **BII6** *m*/*z* calcd (M) 1847.2, obsd 1848.5 (M + H^+); **BII6F2** *m*/*z* calcd (M) 2063.1, obsd 2064.2 (M + H^+); **BII10** *m*/*z* calcd (M) 1477.8, obsd 1479.2 (M + H^+); **BII10F2** *m*/*z* calcd (M) 1693.7, obsd 1695.2 (M + H^+); **M2** *m*/*z* calcd (M) 2476.4, obsd 2496.1 (M + Na^+); **M2F2** *m*/*z* calcd (M) 2692.3, obsd 2693.6 (M + H^+); **M2F5** *m*/*z* calcd (M) 3114.2, obsd 3115.5 (M + H^+).

1 2 3 4 5 6 7 8 9 ..

BII1 NH_2–TRSSRAGLQFPVGRVHRLLRK–CO_2H (+6)

BII1F2 NH_2–TRSSRAGLQFPVGRVHRLLRK–CO_2H (+6)

BII5 NH_2–RAGLQFPVGRVHRLLRK–CO_2H (+5)

BII5F2 NH_2-RAGLQFPVGRVHRLLRK–CO_2H (+5)

BII6 NH_2–AGLQFPVGRVHRLLRK–CO_2H (+4)

BII6F2 NH_2–AGLQFPVGRVHRLLRK–CO_2H (+4)

BII10 NH_2–FPVGRVHRLLRK–CO_2H (+4)

BII10F2 NH_2–FPVGRVHRLLRK–CO_2H (+4)

1 2 3 4 5 6 7 8 9 ..

M2 NH_2–GIGKFLHAAKKFAKAFVAEIMNS–$CONH_2$ (+4)

M2F2 NH_2–GIGKFLHAAKKFAKAFVAELMNS–$CONH_2$ (+4)

M2F5 NH_2–GIGKFLHALKKFLKAFLAELMNS–$CONH_2$ (+4)

Melittin NH_2–GIGAVLKVLTTGLPALISWI KRKRQQ–$CONH_2$

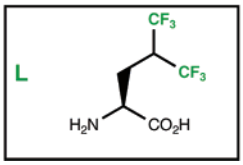

Figure 1. Amino acid sequences of antimicrobial peptides. The net charges at pH 7.40 are given in parentheses. **L**: 5,5,5,5′,5′,5′-2*S*-hexafluoroleucine.

Antimicrobial Activity. Minimal Inhibitory Concentrations (MICs) were measured against Gram-negative *Escherichia coli* (ATCC 23716) and Gram-positive *Bacillus subtilis* (SMY) using mid-logarithmic phase cells. Bacteria from a single colony were grown overnight in Luria Bertani broth at 37 °C with agitation. An aliquot was taken and diluted (1:50) in fresh broth and cultured for ~2 h. The cells (OD_{590} ~0.5) were diluted to a concentration of 5 × 10^4 colony forming units/mL (CFU/mL) for the **BII** series peptides and a concentration of 5 × 10^5 CFU/mL for the **M2** series. Serial dilution (2-fold) of peptide solutions was performed in a sterile 96-well plate (MICROTEST) in duplicate to a final volume of 50 μL in each well, followed by addition of 50 μL of cell suspension. The concentrations of peptides tested were from 0.3 μg/mL to 256 μg/mL. Subsequent to incubation at 37 °C for 6 h, the absorbance at 590 nm was monitored using a microtiter plate reader (VERSAmax). The MIC was recorded as the concentration of peptide required for the complete inhibition of cell growth (no change in absorbance).

Hemolysis Assay. Fresh human red blood cells (hRBCs) were centrifuged at 3500 rpm and washed with PBS buffer until the supernatant was clear. The hRBCs were then resuspended and diluted to a final concentration of 1% (v/v) in PBS and stored at 4 °C. Serial dilution (2-fold) of peptides in PBS in a 96-well plate resulted in a final volume of 20 μL in each well, to which 80 μL of hRBCs were added. The plate was incubated at 37 °C for 1 h, followed by centrifugation at 3500 rpm for 10 min using a SORVALL tabletop centrifuge. An aliquot (50 μL) of supernatant was transferred to a new

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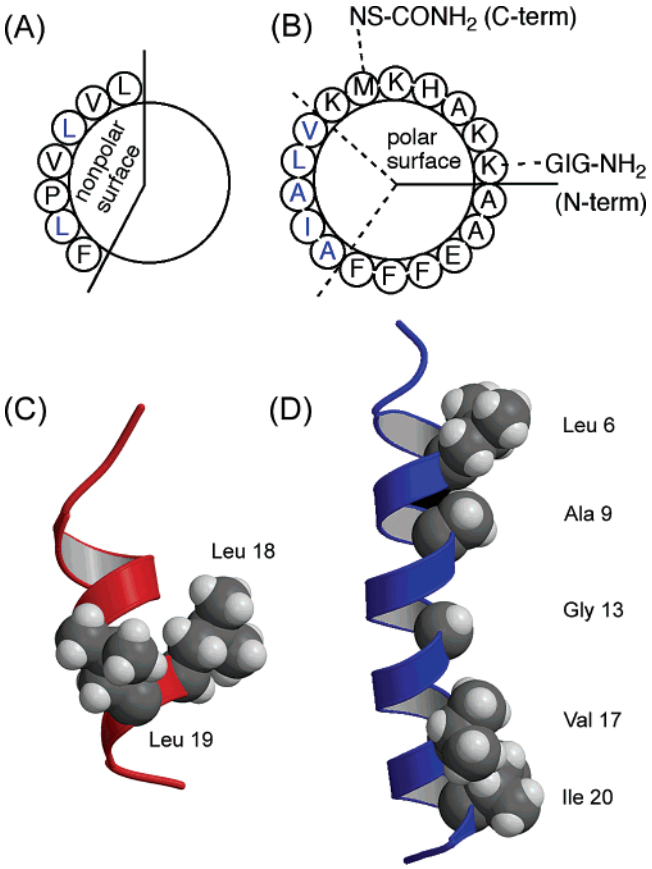

Figure 2. Helical wheel diagrams and sites of fluorination. (A) Buforin series: the arrangement of nonpolar residues is based on ref 38, Leu 18 and 19 (blue) were substituted with hexafluoroleucine; (B) magainin series sites of fluorination: residues Leu 6 and Ile 20 in **M2F2** and Leu 6, Ala 9, Gly 13, Val 17, and Ile 20 in **M2F5**; (C) model structure of **BII10**.[54] Leu 18 and 19 are shown in space-filling depiction; (D) NMR structure of magainin 2 in dodecylphosphocholine micelles (PDB code: 2mag).

Table 1. Antimicrobial and Hemolytic Activities and Hydrophobicities of the Peptides

peptide	MIC[a] (μg/mL) *E. coli*	MIC[a] (μg/mL) *B. subtilis*	HC_{50}[b] (μg/mL)	solvent B (%) for elution[c]
BII1	20	10	400	31.0
BII1F2	5	2.5	400	37.0
BII5	5	5	400	32.1
BII5F2	5	5	400	38.4
BII6	>256	>256	400	33.0
BII6F2	80	20	400	39.5
BII10	>256	>256	400	29.0
BII10F2	40	10	400	35.7
M2	2.5	2.5	175	56.5
M2F2	2.5	2.5	20	62.1
M2F5	40	10	11	85.8

[a] MICs represent at least two independent experiments, each in duplicate, and are reported as the highest determined values. [b]The HC_{50} values were extracted by extrapolation of the fitted curve to 50% lysis of hRBCs. When no detectable hemolytic activity was observed at 400 μg/mL, a value of 400 μg/mL was used. [c]Percentage solvent B (9:1:0.007 $CH_3CN/H_2O/CF_3CO_2H$) required for elution on RP-HPLC (J.T. Baker C_{18}, 5 μ, 4 mm × 250 mm).

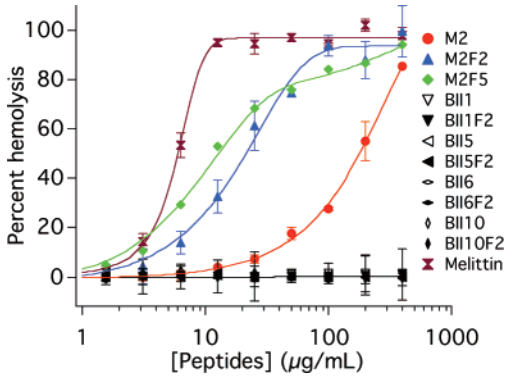

Figure 3. Hemolytic activities of peptides against type B hRBCs relative to melittin. Each data point is the average of at least two independent experiments with two replicates.

96-well plate containing 50 μL of H_2O in each well. Release of hemoglobin was monitored at 415 nm using a microtiter plate reader. Percentage hemolysis was calculated using eq 1:

$$\text{Percentage hemolysis} = 100 \cdot \frac{(A_{415,\text{peptide}} - A_{415,\text{buffer}})}{(A_{415,\text{complete hemolysis}} - A_{415,\text{buffer}})} \quad (1)$$

where complete hemolysis is defined as the average absorbance of all wells containing 400 μg/mL melittin. Negative controls consisted of hRBCs alone.

Protease Stability of Peptides. The proteolytic stability of peptides toward trypsin (from bovine pancreas, EC 3.4.21.4) was determined by an analytical RP-HPLC assay. A standard substrate, *N*-α-benzoyl-L-arginine ethyl ester (BAEE), was used to calibrate enzymatic activity by measuring absorbance at 254 nm. The enzyme concentration (in 1 mM HCl) was determined by absorbance at 280 nm ($\epsilon^{1\%} = 13.3\ \text{cm}^{-1}$). In a typical trypsinization experiment, the buforin peptides (0.2 mM) and 0.1 μg trypsin or the magainin peptides (0.25 mM) and 0.2 μg trypsin in 200 μL of PBS buffer (pH 7.4, 10 mM PO_4^{3-}, 150 mM NaCl) were used. The amount of enzyme was optimized so that the kinetics of proteolytic reactions could be assayed by RP-HPLC (detection at 230 nm). The peptides were incubated with trypsin at 37 °C over a period of 3 h. Aliquots (10 μL) were taken at different reaction times, diluted with 0.2% TFA (440 μL), and stored at −80 °C. A C_{18} analytical column [J.T. Baker C_{18}, 5 μ, 4 mm × 250 mm] was used for separation and quantitation of digested products. The remaining full-length peptide concentration was normalized with respect to the initial concentration. Pseudo-first-order rate constants were obtained by data (<initial 20 min) using eq 2:

$$y = a + b \cdot e^{-k*t} \quad (2)$$

where *y* is the normalized concentration of peptides; *k*, the pseudo-first-order rate constant; *t*, the reaction time in mins; and *b*, the initial concentration of peptides. Each fragment cleaved from the full-length peptides was identified by ESI-MS so that cleavage patterns could be established and compared.

Circular Dichroism. Circular dichroism (CD) spectra were recorded at 25 °C on a JASCO J-715 spectropolarimeter fitted with a PTC-423S single position Peltier temperature controller using a 1 cm path length cuvette. TFE titrations were carried out in PBS buffer at a fixed peptide concentration (10 μM). Four scans were acquired per sample and averaged to improve the *S*/*N* ratio. A baseline was recorded and subtracted after each spectrum. Mean residue ellipticities ([θ], deg·cm²·dmol⁻¹) were calculated using eq 3:

$$[\theta] = \theta_{\text{obs}} \times \text{MRW}/10 \cdot l \cdot c \quad (3)$$

where θ_{obs} is the measured signal (ellipticity) in millidegrees, *l*, the optical path length of the cell in cm, *c*, the concentration of the peptide in mg/mL, and MRW, the mean residue molecular weight (molecular

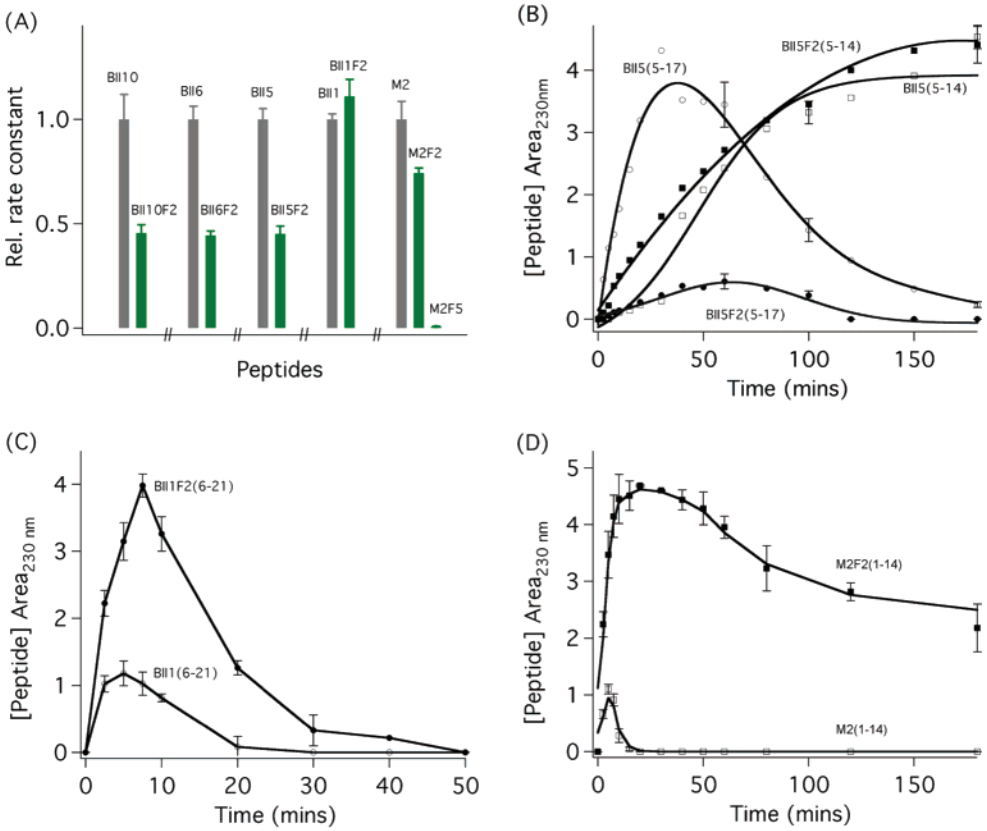

Figure 4. Protease stability as probed using analytical RP-HPLC. Aliquots of reaction mixtures were removed, quenched, and analyzed by RP-HPLC and ESI-MS. (A) Relative rates of proteolytic cleavage of fluorinated peptides compared to hydrocarbon controls; (B) fragments **BII5***(5−14) and **BII5***(5−17) appearance and degradation; (C) fragment **BII1***(6−21) appearance and degradation; (D) fragment **M***(1−14) appearance and degradation. Rate constants for degradation of fluorinated peptides are relative to the parent hydrocarbon peptide. Each data point is the average of two independent experiments.

weight of the peptide divided by the number of residues). Percentage helical contents were calculated using eq 4:

$$\text{Helical content (\%)} = \frac{[\theta]_{222} \times 100}{-40\,000 \cdot \left[1 - \frac{2.5}{n}\right]} \qquad (4)$$

where $[\theta]_{222}$ is the mean residue ellipticity at 222 nm, and n, the number of residues.[31,32]

Analytical Ultracentrifugation. Equilibrium sedimentation analysis was performed for **M2**, **M2F2**, and **M2F5** at 25 °C on a Beckman ProteomeLab XL-I analytical ultracentrifuge. Peptides dissolved in PBS were loaded into equilibrium cells at concentrations of 50 and 100 μM. Absorbance data at 230 nm were acquired at three different rotor speeds (35 000, 40 000, and 45 000 rpm) after equilibration for 18 h. Data obtained were fitted using eq 5 that describes the sedimentation of a single ideal species using Igor Pro v5.03:

$$\text{Abs} = A' \exp(H \times M[x^2 - x_0^2]) + B \qquad (5)$$

where *Abs* = absorbance at radius x, A' = absorbance at reference radius x_0, $H = (1 - \bar{V}\rho)\omega^2/2RT$, $\bar{V}$ = partial specific volume (PSV = 0.7673 mL/g), ρ = density of solvent (1.0017 g/mL), ω = angular velocity in radians/s, R = gas constant in g/mol·K, T = absolute temperature (298 K), M = apparent molecular weight (Da), and B = solvent absorbance (blank). The partial specific volumes of peptides were estimated according to amino acid composition using the program SEDNTERP, assuming the PSV of hexafluoroleucine to be equivalent to that of leucine.

Results and Discussion

Model Peptides and Sites of Fluorination. Two antimicrobial peptides, buforin II (**BII**)[33] and magainin II amide (**M2**),[12] were chosen as templates for fluorination. While both peptides are capable of exerting their bactericidal activity at low micromolar concentrations, their modes of action are quite distinct. Although both are initially drawn to negatively charged bacterial membranes by electrostatic interactions, **M2** causes cell lysis by forming torodial pores in lipid bilayers,[5,7] whereas **BII** penetrates into the cell and kills bacteria presumably by binding intracellular DNA and RNA.[34,35] Furthermore, the peptides chosen are selective against bacterial cells as is evident from their low hemolytic activity. With regard to their potency, while the peptides in the **M2** series operate at the lower limit of MIC concentration typically observed,[4] the **BII** peptides in principle could benefit from increased efficacy. Both translocation of **BII** into cells and pore formation by **M2** seem to be partially controlled by hydrophobic interactions.[7,36,37] The transfer free energies from water to *n*-heptanol for side chains of hexafluoroleucine (−2.5 kcal/mol) and leucine (−2.1 kcal/

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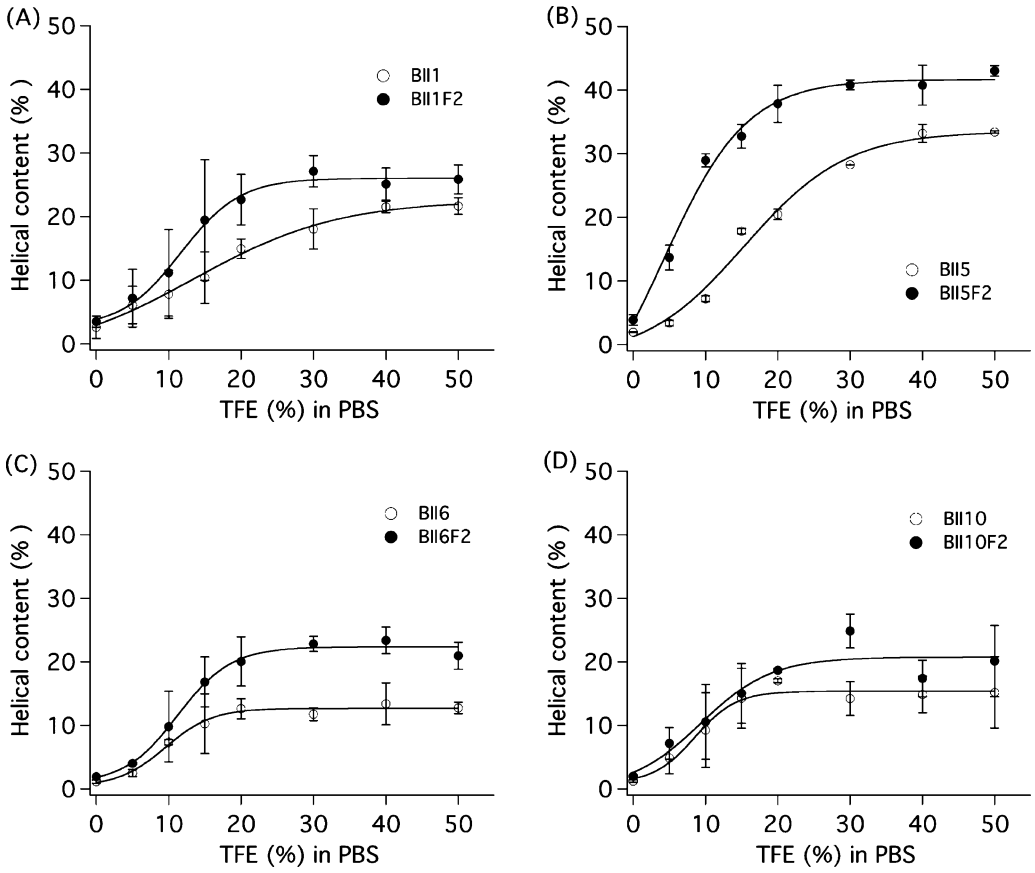

Figure 5. Helical content of buforins and fluorinated analogues in TFE/aqueous buffer probed by circular dichroism spectroscopy. (A) **BII1** and **BII1F2**; (B) **BII5** and **BII5F2**; (C) **BII6** and **BII6F2**; (D) **BII10** and **BII10F2**.

mol)[25] point to the superior hydrophobicity of the fluorinated congener. We envisaged that incorporation of hexafluoroleucine at selected positions would simultaneously increase membrane affinity and provide greater protease stability. We also explored fluorinated derivatives of buforin peptides truncated at the N-terminus. The sequences of peptides and the fluorinated analogues are shown in Figure 1. Since these peptides adopt amphipathic helical conformations, sites of fluorination were selected on the nonpolar face of helices with the help of helical wheel diagrams. In the case of the buforin peptides, leucine residues at positions 18 and 19 on the nonpolar face, which form part of the putative DNA/RNA binding sequence,[34,38] were replaced with hexafluoroleucine. **M2F2** was constructed by replacing Leu6 and Ile20 with hexafluoroleucine. **M2F5** was designed by replacing five residues on the nonpolar face with hexafluoroleucine to yield a highly fluorinated surface (Figure 2).

Antimicrobial Activity. The antimicrobial activity was assessed as a minimal inhibitory concentration (MIC) using turbidity assays against both Gram-positive (*B. subtilis*) and Gram-negative (*E. coli*) bacteria (Table 1). All of the fluorinated peptides had better or similar antimicrobial activities relative to the parent peptides with the exception of **M2F5**. Three of the four fluorinated buforin analogues had increased potency, with improvements in MIC values ranging from 3-fold to greater than 25-fold, while the fourth derivative (**BII5F2**) had equivalent activity as compared to the control peptides. On the other hand, **M2F2** exhibited same MIC values as **M2** while **M2F5** was 4- and 16-fold less active against *B. subtilis* and *E. coli*. A survey of the antimicrobial host defense peptides suggests that the most active members have MICs in the 1−3 μM range.[4,39,40] It is therefore not surprising that **BII5F2** and **M2F2** show essentially the same efficacy as **BII5** and **M2** as these peptides are already operating at the potency limit. Furthermore, the data obtained demonstrate that the bioactivity of these peptides is either retained at the frequently invoked maximal potency or improved by means of fluorination. Selective, controlled fluorination may therefore serve as a powerful tool to enhance the activity of known antimicrobial peptides to optimal levels. The only peptide to have lost its potency upon fluorination, **M2F5**, forms a helical bundle in aqueous solution (*vide infra*).

Hemolytic Activity and Hydrophobicity. The ability of the peptides to disrupt the membrane integrity of mammalian cells was interrogated by a hemolysis assay using type B hRBCs.[13] The buforin analogues had hemolytic activity essentially the same as those associated with the control peptides indicating that passage across the membrane was not compromised by fluorination (Figure 3). In addition, the data are also indicative of the fact that these peptides do not operate by membrane lysis

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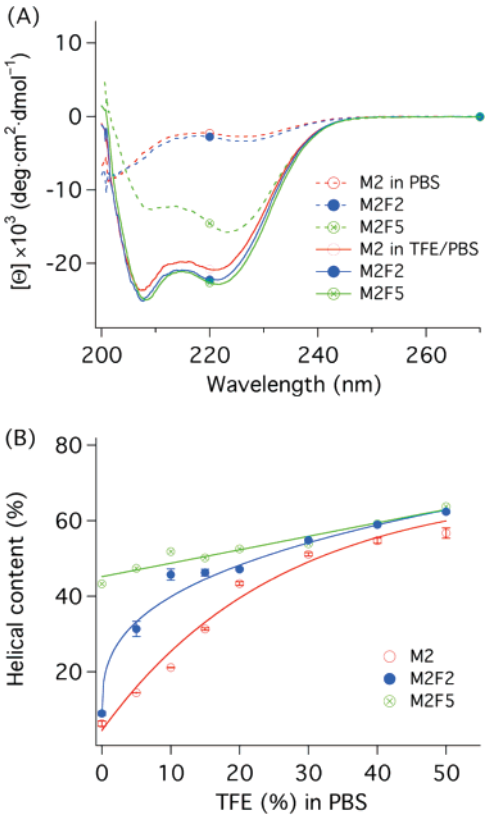

Figure 6. Circular dichroism spectra and effect of TFE on the helical content for magainins. (A) CD spectra of **M2**, **M2F2**, and **M2F5** in PBS and 50% TFE in PBS; (B) TFE effect on the helical content of **M2**, **M2F2**, and **M2F5**.

as is the case in the magainin series. On the other hand, **M2F2** was moderately more hemolytic than **M2** at the MIC value[41] (Figure 3).

It has been demonstrated previously that increased hydrophobicity correlates with hemolytic activity.[4,36,42] Accordingly, we evaluated the hydrophobicity of the peptides based on their retention times on reversed phase HPLC.[26,36] The introduction of hexafluoroleucine resulted in increased hydrophobicity of parent peptides with an average increase of 3.1(±0.2)% solvent B per Leu (or Ile) substituted. The two peptides exert their action using fundamentally different mechanisms, and it appears that increased hydrophobicity correlates with greater antimicrobial activity in the buforin series, with essentially no perturbation of hemolytic potential. On the other hand, the magainin derivatives show a significant increase in the ability to disrupt hRBC membranes. Overall, these results are consistent with previous studies that showed increased hydrophobicity results in higher hemolytic tendency, indicating that a fine balance between hydrophobicity and charge as in the case of magainin-like peptides is crucial for redesign with fluorinated analogues. It is likely that a hydrophobicity maximum of the parent peptide (>75% Solvent B required for elution in RP-HPLC under the conditions specified in Table 1) exists, beyond which fluorination may not result in retention of selectivity for bactericidal activity over mammalian cell permeabilization.

Protease Stability. A major limitation of antimicrobial peptide function is their inactivation by proteases. We examined the susceptibility of the various peptides against trypsin,[14,43] a commercially available protease. Trypsin specifically catalyzes the hydrolysis of C-terminal amide bonds of lysine and arginine, making it an ideal enzyme to examine this parameter as the substrates used in our study contain multiple cleavage sites in the form of residues with cationic side chains. Poor protease stability severely limits the clinical use of many therapeutic peptides.[44,45] Results reported here will therefore inform future investigations involving fluorination of peptide-based drug candidates.

All fluorinated peptides were generally more stable toward trypsin digestion (Figure 4). The **BII** analogues **BII5F2**, **BII6F2**, and **BII10F2** were ~2.3-fold more resistant to hydrolysis, while **BII1F2** was similar to **BII1**. The initial P1 site of cleavage, according to the nomenclature of Schechter and Berger,[46] was different in **BII5F2** (R14) than **BII5** (R17). This was also the case when one compares the initial cleavage site in **BII1F2** and **BII1** (See Supporting Information). In addition, the initial cleavage fragment **BII1F2**(6−21) accumulated and persisted much longer than **BII1**(6−21). In both cases, the presence of hexafluoroleucine at the P1′ and P2′ sites confers solid protection to the R17 cleavage site. In addition, **BII6F2** is a much more effective antimicrobial than **BII6**, which may make **BII1F2** retain potency even after suffering proteolytic degradation. A similar trend was observed for the magainin analogues. **M2F2** was more stable to proteolysis relative to **M2** by 35%, whereas **M2F5** was fiercely resistant to degradation, with >78% of the peptide remaining in solution after 3 h. In contrast, **M2** is completely hydrolyzed in less than 40 min. The initial fragment resulting from cleavage of **M2F2**(1−14) accumulated in higher amounts than **M2**(1−14) and only underwent minimal proteolytic degradation over 3 h. The presence of a single hexafluoroleucine residue at position 6 (P2′ site) in **M2F2**(1−14) confers a dramatic advantage in protecting the K4 amide bond. Collectively, hexafluoroleucine at either the P1′ site and/or P2′ site is sufficient to provide protection against catalyzed hydrolytic damage.

The mechanism by which hexafluoroleucine is able to impart greater protease stability requires further detailed investigation. To a first approximation, the effect could either be attributed to the increased steric bulk of the fluorinated amino acid or to electronic perturbation of the amide bond. The acid dissociation constants for hexafluoroleucine (pK_a values of 7.51 and 2.79) indicate that the acidity of ammonium group is increased ~100 fold compared to leucine (pK_a values of 9.71 and 2.61).[47] The substitution of a single fluorine for hydrogen is often considered to be isosteric; however, the side chain volume of hexafluoroleucine is calculated to be ~37 Å^3 larger than that of leucine.[25] Taken together, the evidence suggests that while a moderate electronic perturbation may still be operational, it is more likely that the protease protection is a result of steric occlusion of the peptide from the active site. In the case of **M2F5**, the

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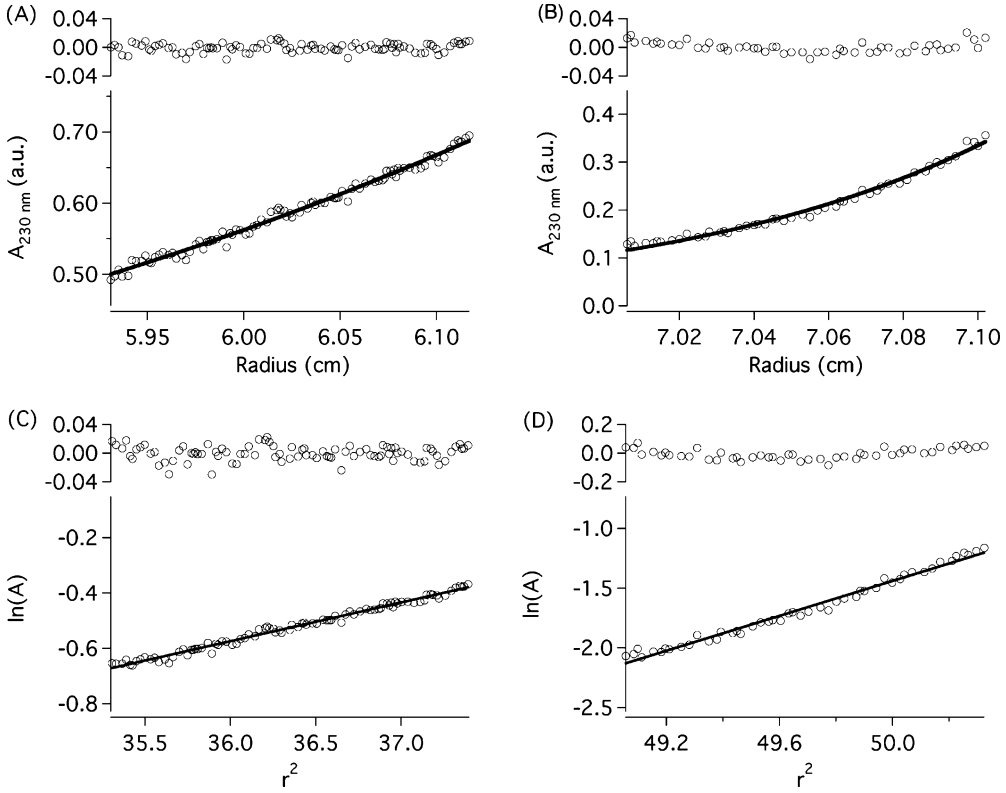

Figure 7. Representative equilibrium analytical ultracentrifugation traces for **M2** (A) and **M2F5** (B) [25 °C, 35000 rpm at 230 nm]. Fits to a single ideal species model are shown as a solid line with residuals in the top frame. Conditions: [peptide] = 50 μM, 10 mM phosphate, pH 7.40, 137 mM NaCl, 2.7 mM KCl. The observed apparent molecular weights were 2413 (**M2**, calc. 2478 for monomer) and 12 436 (**M2F5**, calc. 12 460 for tetramer). Plots of ln(A) vs r^2 for **M2** (C) indicate a single ideal species, while nonrandom residuals for **M2F5** (D) suggest presence of other aggregation states.

conformational stability is greatly increased, thereby denying the protease access to the labile amide.

Circular Dichroism and Analytical Ultracentrifugation. Antimicrobial potency has been shown to correlate closely with α-helical content in several instances. Accordingly, we used CD spectroscopy to probe the influence of fluorination on secondary structure formation in TFE/water mixtures, a solvent known to mimic the nonpolar environment of membranes. All peptides with the exception of **M2F5** were unstructured in aqueous solutions. However with increasing amounts of TFE, the peptides adopted an α-helical structure, consistent with their putative membrane active conformations. The fluorinated peptides exhibited an increased propensity for adopting α-helical conformations in both the buforin and magainin series peptides (Figures 5 and 6). In the case of the buforin peptides, higher α-helical content is coupled closely with antimicrobial potency.

At 50% TFE, **M2**, **M2F2**, and **M2F5** were ∼60% helical. However, only **M2F5** was significant helical (∼40%) in buffered aqueous solutions with no exogenous TFE (Figure 6). This dramatic difference necessitated further examination of the respective oligomeric states. **M2** was monomeric as judged by analytical ultracentrifugation while both **M2F2** and **M2F5** had a tendency to populate multiple oligomeric states (Figure 7 and Supporting Information). Indeed, **M2F5** appears to form stable helical bundles in aqueous solutions providing an explanation for decreased antimicrobial activity, increased hemolytic activity, and greatly enhanced protease stability.[32,48,49]

Overall, the α-helical content is related to more pronounced antibacterial action in the case of buforins. In contrast, higher amounts of secondary structure correlates with increased hemolysis and formation of aggregates in the magainin series.

Conclusions

Stabilization of helical peptide assemblies by incorporation of fluorinated amino acids has recently been realized by several groups.[21−24,50,51] However, incorporation of such amino acids into bioactive peptides has been limited.[52,53] We have demonstrated here that incorporation of hexafluoroleucine at selected sites in antimicrobial peptides results in simultaneous enhancement of potency and increased resistance to protease degradation. Five of six fluorinated peptides (**M2F2**, **BII1F2**, **BII5F2**, **BII6F2**, and **BII10F2**) used in this study showed significantly enhanced (or similar) bactericidal activity. The lone exception, **M2F5**, which is highly fluorinated, had a proclivity to form helical bundles in aqueous solutions and had diminished antimicrobial power and increased hemolytic activity. Furthermore, this peptide was extremely resistant to protease action. The other five analogues showed increased or similar protease stability as well as altered cleavage

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patterns. The hemolytic properties of these peptides were comparable to the parent control peptides from which they were derived. Poor protease stability of short α-peptides severely limits their therapeutic value. Our study highlights the utility of fluorinated peptides in reviving promising leads that suffer such a fate. Further, it is evident that one could perturb peptide−receptor interactions using fluorinated derivatives.

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Supporting Information Available: HPLC data detailing protease stability assays and accompanying kinetic analysis. ESI-MS data for all peptide fragments generated in protease degradation assays, circular dichroism spectra, and AUC data for peptides in tabular format. This material is available via the Internet at http://pubs.acs.org/.

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