Application of 3D NMR for Structure Determination of Peptide Natural Products

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ABSTRACT: Despite the advances in NMR, structure determination is often slow and constitutes a bottleneck in natural products discovery. Removal of this bottleneck would greatly improve the throughput for antibiotic discovery as well as other therapeutic areas. Overall, faster structure methods for structure determination will serve the natural products community in a broad manner. This report describes the first application of 3D NMR for elucidation of two microbially produced peptide natural products with novel structures. The methods are cost-effective and greatly improve the confidence in a proposed structure.

INTRODUCTION

Drug resistant infectious diseases, in particular Gram-negative infections, continue to threaten global health as well as contemporary medical practices.1 For example, as pathogens become increasingly resistant, semi-routine procedures such as organ transplants and joint replacements will become increasingly dangerous. Combined with the emergence of pan-resistant pathogens, new antibiotics are sorely needed. Although natural products have provided the antibiotic basis upon which many medical practices were built, discovering novel antibiotics has become more challenging. Nonetheless, advances in sequencing and genomics has not only indicated that there is more potential than previously thought, but also advances in mass spectrometry as well as the speed and resolving power of UPLC systems have enabled rapid identification of novel antibiotics.2–5 In parallel, analytical strategies that take advantage of increasing sensitivity of mass spectrometers as well as the speed and resolving power of UPLC systems have enabled rapid identification of novel antibiotics.6–12 Combining these new tools with the bacterial diversity found in underexplored ecological niches such as insect symbionts,13 thermal vents,14 caves,15 coal mines,16 and marine ecosystems17 has demonstrated great promise for discovering the next generation of antibiotic therapies.

To address the challenges of resistant infectious disease, the potential of these genetic and analytical methods must be fully realized. Our lab has focused on LCMS-based metabolomics as a discovery platform to discover novel natural products from marine invertebrate-associated bacteria. This has led to a high discovery rate of novel natural products.9–11 As these methods pave the way for rapid discovery, structure determination becomes a limiting factor for providing valuable lead structures. Therefore, we have developed methods such as isotopic labeling followed by 13C–15N gCOSY11,18,19 for rapid assembly of carbon backbones in polyketides and 13C–15N NMR11 to assist with thiazolines and other heterocyclic systems. While our previous studies demonstrated a high level of 13C enrichment could be achieved for molecules of acetate origin, questions remained surrounding isotopically labeling peptides. For example, typical growth media used were rich in natural abundance precursors such as amino acids from sources like yeast extract and peptone. On the other hand, if uniform labeling could be achieved for both 13C and 15N, structure determination of peptide-based natural products could be achieved using biomolecular NMR strategies such as 3D NMR techniques. These approaches would also provide a gateway to automated backbone assignment of structures containing typical amino acids. Therefore, we evaluated isotopic labeling in a medium that contained typical quantities of nonenriched components. To demonstrate proof of concept for both isotopic labeling and 3D NMR strategies, we tested the methods using two novel antibiotic peptides, one of which was prioritized as part of an antibiotic drug discovery program. In particular, peptide 2 showed broad spectrum activity including against Gram-negative pathogens. Although peptide 2 did not show high potency, the difference between the MIC (22.7 μM) and MBC (45.5 μM) was small indicating potential for Gram-
RESULTS AND DISCUSSION

Uniform Labeling Peptides with $^{13}$C and $^{15}$N. Our previous studies showed that fermentation of actinobacteria upon addition of uniformly $^{13}$C-labeled glucose and subsequent purification yielded $^{13}$C-labeled natural products. However, peptides could be more challenging since in most cases U-13C glucose was utilized for $^{13}$C enrichment in natural products which have been biosynthesized via a polyketide pathway. For the $^{15}$N incorporation, $^{15}$NH$_4$Cl was evaluated as the nitrogen source. As mentioned, a potential issue was $^{15}$N incorporation with peptides in rich media. To evaluate the $^{13}$C and $^{15}$N incorporation, a marine invertebrate-derived myce (strain WMW 705) that produced two novel peptides that we named eudistamides A (1) and B (2) was grown in multiple media and extracts were analyzed by LC–MS data (Supporting Information). Although the medium containing $^{15}$NH$_4$Cl as the only nitrogen source (M3, Supporting Information) yielded the highest $^{15}$N abundance of the peptides, taking into consideration the various factors such as the price of the medium, the yield, and the $^{13}$C and $^{15}$N abundance, the labeled medium containing peptone and yeast extract (20 g soluble starch, 10 g [U-$^{13}$C] glucose, 2.5 g peptone, 2.5 g yeast extract, 5 g $^{15}$NH$_4$Cl, 5 g CaCO$_3$/L of artificial seawater) was used for producing $^{13}$C- and $^{15}$N-labeled peptides.

Strategies and Experiments for the Resonance Assignment of Uniformly $^{13}$C, $^{15}$N-Labeled Peptides. Backbone Assignment Strategy. A typical triple resonance 3D NMR spectrum observes correlations of three resonances, a proton, a carbon, and a nitrogen. Many 3D NMR experiments have been designed for assigning the backbone of $^{13}$N/$^{15}$C isotopically labeled protein, such as HNCO,$^{21}$ NH(CA)CO,$^{22}$ HNCA,$^{23}$ CBCANH,$^{23}$ CBCA(CO)NH,$^{24}$ HN(CO)CA,$^{25}$ HCACO,$^{21}$ HCA(CO)N,$^{21}$ H(CCO)NH,$^{26}$ C(CO)NH,$^{26}$ and HBHACBCACO)NH.$^{27}$ Ideally, standard triple resonance backbone assignment of a peptide has relied on a combination of the CBCANH, CBCA(CO)NH, and NHCO spectra (Figure 1a–c). The general idea was that the CBCANH correlated each NH group with the C$_{\alpha}$ and C$_{\beta}$ chemical shifts of its own residue (strongly) and of the preceding residue (weakly), whereas the CBCA(CO)NH only correlates the NH group to the preceding C$_{\alpha}$ and C$_{\beta}$ chemical shifts. The NHCO experiment provides the connectives between each NH group with the carbonyl carbon of the same residue, thus establishing the backbone assignment. In our experience, the quality of the CBCANH and CBCA(CO)NH spectra was sometimes not sufficient in terms of S/N. The C$_{\alpha}$ or C$_{\beta}$ resonances were, for example, not visible above the noise level. In this case, the combination of HNCA and HN(CO)CA experiments (Figure 1d–f) which were more sensitive provided the same information as the CBCANH and CBCA(CO)NH spectra, except without the C$_{\beta}$ resonances. The HN(CA)CO experiment (Figure 1d) was powerful for linking each NH group with the carbonyl carbon of the same residue, particularly helpful to the assignment of depsipeptides. A depsipeptide is a peptide in which one or more of its $\text{C(O)NR}$ groups are replaced by the corresponding ester, $\text{C(OR)}$. Taking eudistamides A (1) and B (2) for example, no correlation for C-1 could be seen in NHCO spectrum, whereas HN(CA)CO experiment could correlate NH-7 with...

![Figure 1](http://example.com/image.png)
the carbonyl carbon of the ester (C-1). Therefore, our backbone assignment strategy used three 3D NMR experiments, HNCO, CBCANH, and CBCA(CO)NH, to establish the connectivity of the backbone as the first step. The other 3D NMR experiments, NHCA, HN(CO)CA, NH(CA)CO, were utilized to make further confirmation regarding to some complicated cases, such as overlapping carbonyl carbon signals and any crosspeak that was very weak or missing in either CBCANH or CBCA(CO)NH spectrum. We were thereby able to make unambiguous backbone assignment of the eudistamides A and B and provided a general strategy for non-Pro containing peptide natural products. However, due to the absence of an amide proton in proline, these 3D NMR experiments cannot be used to sequentially connect proline residue to the preceding residue. The CDCA(NCO)CAHA (Figure 1h) was designed for the sequential assignment of proline residues, and the other option is to rely on the observation of NOEs to neighboring residues.

**Side Chain Assignment Strategy.**

Depending on the structure, side chains can be assigned using TOCSY and HSQC. However, the most useful NMR experiment for side chain assignment is an HCCCH-TOCSY spectrum (Figure 1g). The general principle behind using the HCCCH-TOCSY spectrum is as follows: Using the known Cα and Cβ chemical shifts from the backbone assignment, the side chain chemical shifts would be assigned by viewing 2D slices at each carbon shift, typically at the Cα and Cβ chemical shifts. Each "slice" contains resonances for the protons attached to carbons in that 13C−15N spin system. The HCCCH-TOCSY experiment combined with 13C chemical shift ranges of the common amino acids provides a very powerful means for the assignment of the aliphatic side chain. Some of the proteinogenic amino acids, such as alanine, serine, threonine and glycine, were easily identified since alanine, serine, and threonine’s Cβ chemical shifts were unique compared to those of the other amino acids and glycine has no Cα. Valine, isoleucine, and proline could also be easily identified because they have lower than normal Cα chemical shifts. For the aromatic side chain assignment, such as tyrosine, phenylalanine, tryptophan, and histidine, additional 2D NMR data are needed to complete the whole side chain assignment.

**Structure Elucidation of Eudistamides A (1) and B (2).** Eudistamide A (1) was obtained as a white powder with a molecular formula of C72H99N11O18 as determined by HRESIMS. Interpretation of HCCCH-TOCSY data (Table S1; Supporting Information) of the 13C- and 15N-doubly labeled 1 established the amino acid residues leucine (Leu; 2×), arginine (Arg), proline (Pro), glycine (Gly), glutamine (Gln), alanine (Ala), valine (Val), and phenylalanine (Phe), which were further confirmed by natural abundance experiments, 1H−1H COSY, HSQC and HMBC data (Table S3; Supporting Information). The Pro residue was assigned as cis based on the 13C NMR chemical shifts of the β- and γ-carbon atoms (Δδβγ). Δδβγ for trans-Pro is regularly less than 5 ppm, while Δδβγ for cis-Pro is regularly between 5 and 10 ppm. 15A Δδβγ for the proline residue in 1 was 5.8 ppm, supporting the assignment as cis. The 3-(2-methylphenyl)acrylic acid (Me-acyl) moiety was established by HMBC, whereas geometry of the double bond (C6α and C6β) was assigned as E on the basis of large coupling constant (15.5 Hz) through the double bond.

The connectivity of the partial structures Thr-Phe-Leu2-Val-Ala-Gln-Gly and Pro-Arg-Leu1 was established by using 3D NMR data which were discussed for the backbone assignment (CBCANH, CBCACONH, NHCO, HNCA, NH(CO)CA, NH(CA)CO; Table S1; Supporting Information) of the 13C- and 15N-doubly labeled 1. The HMBC correlation from H-62 to C-1 and the ROESY correlation between H-1 and H-2 connected Leu1 to Thr via an ester on the basis of the chemical shift of C-62 (δc 68.5). Moreover, for the uncommon amino acid residue, the HMBC correlations from H-66 and H-67 to C-65 supported the linkage of Me-acyl moiety to Thr. The ROESY correlations between H-24a and H-22b secure the ring closure by connecting the Gly unit to Pro, which satisfied the 24 degrees of unsaturation deduced from the molecular formula.

The advanced Marfey’s method11 was applied to assign the absolute configurations of the amino acid residues from acid hydrolysis of 1. The 1-fluoro-2,4-dinitrophenyl-5-leucine-amide (FDLA) derivatives of the hydrolysate of 1 and authentic D- and L-amino acids were subjected to LC−MS analysis. The absolute configuration of all amino acids was established by comparison of their HPLC retention time and molecular weights with those of corresponding authentic D- and L-standards. Upon analysis, the amino acid residues Arg, Pro, Ala, Val, allo-Thr, and Phe were determined to have the L-configuration, whereas the Gln and Leu were deduced to have the D-configuration (Table S5; Supporting Information). Eudistamide B (2) was isolated as a white amorphous powder with a molecular formula of C61H90N14O13, determined by HRESIMS. A detailed analysis of HCCCH-TOCSY (Table S2; Supporting Information) of the 13C- and 15N-doubly labeled 2 indicated an amino acid content as follows: Thr (x2), Tyr (x2), Ser, Lys, 3-OH-Leu (x2), Leu, and Phe. CBCANH, CBCA(CO)NH NHCO, HNCA, NH(CO)CA, and NH(CA)CO spectra (Table S2; Supporting Information) revealed the amino acids sequence as 3-OH-Leu-Thr-Tyr-Ser-3-OH-Leu-Lys-Tyr-Leu-Phe-Thr2, which was further confirmed by ESI+MS fragmentation analysis (Figure S2; Supporting Information). The linkage of Me-acyl moiety to Thr3 was supported by the HMBC correlation from H-76 to C-74. The ROESY correlation between H-72 and H-2 and consideration of unsaturation deduced from the molecular formula connected 3-OH-Leu to Thr3 via an ester on the basis of the chemical shift of C-71 (δc 68.5).

The absolute configurations of the amino acids were determined using acid hydrolysis followed by the advanced Marfey’s method. The chromatographic comparison between Marfey’s derivatives of the hydrolysate of 2 and appropriate amino acid standards assigned the L-configuration for Ser and Leu and D-configuration for Lys, Phe, allo-Thr, and Tyr (Table S4; Supporting Information). The (2R, 3S) and (2R, 3R)-3-hydroxylucine were synthesized following Bonnard’s procedure12 and derivatized with L- and L/D-FDLA respectively. Since enantiomers would exhibit identical retention behavior under nonchiral HPLC conditions, the (2R, 3S)-δ-FDLA derivative showed the same retention time as (2S, 3R)-δ-FDLA, whereas the (2R, 3R)-δ-FDLA derivative showed the same retention time as (2S, 3S)-δ-FDLA. By comparing the above the four retention times of the 3-OH-Leu standards with the L-δ-FDLA derivatives of the hydrolysate of 2, both the 3-OH-Leu in 2 were assigned as 2S, 3R (Table S6; Supporting Information). The 2S, 3R configuration of the two 3-hydroxylucines in 2 were also confirmed by J-based analysis and NOESY data. The large coupling constants observed from DQF-COSY in MeOD between H-2 and H-3 (11.5 Hz), and between H-28 and H-29 (11.0 Hz) established their anti-
relationship. The ROESY correlation between H-4 and NH-7, as well as the ROESY correlation between H-30 and NH-33, established the rotamer (A4) in Figure 2.

![Figure 2](image1.png)

Figure 2. J-based configuration analysis of C-2 and C-3 of compound 2.

Compounds 1 and 2 were tested for antibacterial activity against *Pseudomonas aeruginosa*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, and *Bacillus subtilis*. Compound 2 showed antibacterial activity against MRSA, *E. coli* and *B. subtilis*, with the MIC values of 22.7, 22.7, and 2.8 μM, respectively, while compound 1 did not show any detectable antibacterial activities at 52.2 μM. Compound 2 was also tested using a murine thigh model infected with either *E. coli* ATCC 25922 or *S. aureus* ATCC 25923 (Figure 3). Over the 16-fold intraperitoneal dose range, no toxicity associated with compound administration was observed (Figure 3a,c). Conversely, intravenous dose levels above 8 mg/kg resulted in animal death (Figure 3b,d). Modest, but broad spectrum (both Gram-positive and Gram-negative bacteria) efficacy was observed with two highest tolerated dose levels given by both administration routes. Compared to untreated control animals, compound 2 reduced the organism burden in the thighs of immunocompromised mice by more than 0.5 log_{10} cfu/thigh.

Although the cyclopetides and cyclodepsipeptides have been encountered frequently as bacterial secondary metabolites, eudistamides A (1) and B (2) differ markedly from the known peptides by virtue of the presence of a unique 3-(2-methylphenyl)acrylic acid moiety, which has not been previously reported in a peptide. Only some analogues of 3-(2-methylphenyl)acrylic acid residue were rarely described in peptides, such as WS9326s, a pepticinamids, and mohangamides incorporating a 2-pentenyl cinnamic acid moiety, skyllamycins A and B, incorporating a 2-propenyl]-cinnamic acid moiety, and corprisamides A and B bearing 2-heptatrienyl cinnamic acid moiety. Moreover, another remarkable feature of compound 2 is the high content of the β-hydroxy-α-amino acids, including genetically coded proteinogenic amino acids Ser and Thr, and nonstandard amino residue 3-OH-Leu. Members of the β-hydroxy-α-amino acid class occur as constituents of many important antibiotics, such as vancomycin and lysobacin. Furthermore, compounds 1 and 2 could be produced in sufficient quantities by scale-up microbial fermentation, which would greatly facilitate follow-up mechanistic and preclinical studies.

## CONCLUSION

The introduction of 3D NMR has dramatically improved the speed and reliability of the protein assignment process. However, we expanded the scope of 3D NMR experiments from protein applications to a strategy that is universally applicable for labeled peptides. We have demonstrated a rapid and efficient structure elucidation protocol for complex peptides containing typical amino acids. The protocol we propose consists of a typical set of 3D NMR experiments for backbone and side chain assignment of uniformly labeled peptides with 13C and 15N. While 3D NMR experiments may not be optimal for natural product peptides, this proof of concept provides a basis for using triple resonance strategies for rapidly establishing the backbone of peptide natural products. Development of specific NMR experiments would be advantageous. The triple resonance experiments used in this study were highly complementary to more traditional methods such as HMBC and ROESY. This report describes the first application of 3D NMR to two microbially produced peptide natural products with novel structures. The methods are cost-effective and greatly improve the confidence in a proposed structure.

## EXPERIMENTAL SECTION

### General Experimental Procedures

Optical rotations were measured on a Polarimeter. UV spectra were recorded on a UV–vis Spectrophotometer. IR spectra were measured with a FT-IR Spectrophotometer. 1D and 2D NMR spectra were obtained in DMSO with a NMR spectrometer equipped with a 1H/13C/15N cryoprobe. 3D NMR spectra were obtained in DMSO with a NMR spectrometer with a 1H/13C,15N) cold probe. HRMS data were acquired with a QTOF mass spectrometer (Ionization method: Spray Voltage (+) 4000; Spray Voltage (−) 3700; Capillary temperature 320; full MS scan 200–2000). MSMS data were acquired with a hybrid quadrupole-Orbitrap mass spectrometer. RP HPLC was performed using a HPLC system and a Phenomenex Luna C18 column (250 × 10 mm, 5 μm), as well as a preparative HPLC and Phenomenex Gemini
C18 column (250 × 30 mm, 5 μm). The Advanced Marfrey’s method utilized a HPLC coupled with a mass spectrometer.

**Biological Material.** Ascidian specimens were collected in September 2011 from the Florida Keys (24°33'416", 81°21'611"). Identification was confirmed by Shirley Parker-Nance. A voucher specimen for *Eudistoma olivaceum* (Van Name, 1902) is housed at the University of Wisconsin—Madison. For cultivation, a sample of ascidian (1 cm³) was rinsed with sterile seawater and macerated using a sterile pestle in a microcentrifuge tube, and dilutions were made in sterile seawater, with vortexing between steps to separate bacteria from heavier tissues. Dilutions were separately plated on three media: ISP2 supplemented with artificial seawater, R2A, and M45. Each medium was supplemented with 50 μg/mL cycloheximide and 25 μg/mL nalidixic acid. Plates were incubated at 28 °C for at least 28 days, and strain WMMB 705 was purified from an ISP2 isolation plate.

**Sequencing.** Genomic DNA was extracted using the UltraClean Microbial DNA Isolation kit (Mo Bio Laboratories, Inc.). 16S rDNA genes were amplified using 100–200 ng genomic DNA template with the primers 8–27F (5’ to 3’ GAGTTGATCCTGCGTCAAG) and 1492R (5’ to 3’ GGTATCCTTGTAGACCTT). The following PCR conditions were used: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1.5 min, with a final step of 72 °C for 5 min. The PCR bands were excised from the gel and purified using the QIAquick Gel Extraction kit (QIAGEN). One microliter of purified product was sequenced. Sequencing reactions were performed by the UW Biotechnology Center and reactions were sequenced with an ABI 3730xl DNA Analyzer. WMMB 705 were identified as *Streptomyces* sp. FXJ6.141 (accession number GU 1492R (5° for antibacterial activity against MRSA (ATCC 27853), and strain WMMB 705 was purified from an ISP2 isolation plate.

**Fermentation, Extraction, and Isolation.** Two 10 mL of seed cultures (25 × 150 mm tubes) in medium ASW-A (20 g soluble starch, 10 g glucose, 5 g peptone, 5 g yeast extract, 5 g CaCO₃ per liter of artificial seawater) were inoculated with strain WMMB 705 and shaken (200 rpm, 28 °C) for 4 days. Two-liter flasks (2 × 500 mL) containing ASW-A medium with Diaion HP20 (7% by weight) were inoculated with 8 mL from the culture tube and shaken at 200 rpm and 28 °C for 7 days. Filtered HP20 was washed with water and extracted with acetone. The acetone extract was chromatographed on Diaion HP20ps, eluting sequentially with methanol/water (10:90 and 95:5). The 95% methanol eluate was evaporated to dryness to afford a yellow oil (460 mg), and then it was subjected to RP HPLC (30%/70% to 60%/40% ACN/H₂O with H₂O containing 0.1% acetic acid over 30 min, 25 mL/min, followed by 90%/10% to 100% of the same solvents for 5 min, 25 mL/min, and a hold at 100%/0% of the same solvents) using a Phenomenex Gemini C18 column (250 × 30 mm, 5 μm), yielding a fraction containing 1 (50 mg, tₚ 11.5 min), and compound 2 (21 mg, tₚ 12.5 min). The fraction containing 1 was further subjected to RP HPLC (55–65% MeOH–H₂O with H₂O containing 0.1% acetic acid over 25 min, 4.0 mL/min) using a Phenomenex Luna C18 (250 × 10 mm, 5 μm), yielding 1 (22 mg, tₚ 19 min). For 16C and 18N incorporation, the same procedure was used (1 × 250 mL) with labeled medium ASW-A (20 g soluble starch, 10 g U¹³C-glucose, 2.5 g peptone, 2.5 g yeast extract, 5 g NH₄Cl, 5 g CaCO₃ per liter of artificial seawater).

**Antibacterial Assay.** Eudistasinides A (1) and B (2) were tested for antibacterial activity against MRSA (ATCC #33591), *E. coli* (ATCC #25922), *B. subtilis*, and *P. aeruginosa* (ATCC #27853), and MICs were determined using a dilution antimicrobial susceptibility test for aerobic bacteria. ¹³C and ¹⁵N incorporation, the same procedure was used (1 × 250 mL) with labeled medium ASW-A (20 g soluble starch, 10 g U¹³C-glucose, 2.5 g peptone, 2.5 g yeast extract, 5 g NH₄Cl, 5 g CaCO₃ per liter of artificial seawater).

**Synthesis of (2R,3S)-3-Hydroxylysine and (2R,3R)-3-Hydroxylysine and Advanced Marfrey’s Analysis of 3-Hydroxylysines.** (2R,3S)-3-Hydroxylysine and (2R,3R)-3-hydroxylysine were synthesized using the procedures by Bonnard et al. They obtained 0.01 mg of major precursor (S)-1-((2R,SS)-5-isopropyl-3,6-dimethoxy-2,5-dihydroprazin-2-yl)-2-methylpropan-1-ol and 2.5 mg of minor precursor (R)-1-((2S,SS)-5-isopropyl-3,6-dimethoxy-2,5-dihydroprazin-2-yl)-2-methylpropan-1-ol. The hydrolysates were hydrolyzed following the general peptide acid hydrolysis process. The major precursor gave a mixture of (2R,3S)-3-hydroxylysine and D-valine, and the minor precursor gave a mixture of (2R,3R)-3-hydroxylysine and D-valine. These two hydrolysates were derivatized with t-FDLA and d-FDLA, respectively, resulting in four products: (2R,3S)-3-hydroxylysine-t-FDLA, (2R,3S)-3-hydroxylysine-d-FDLA, (2R,3R)-3-hydroxylysine-t-FDLA, and (2R,3R)-3-hydroxylysine-d-FDLA. Among these four products, (2R,3S)-3-hydroxylysine-d-FDLA will show the same retention time as (2S,3R)-3-hydroxylysine-t-FDLA, and (2R,3R)-3-hydroxylysine-t-FDLA will show the same retention time as (2S,3S)-3-hydroxylysine-t-FDLA, under the nonchiral HPLC condition we used in advanced Marfrey’s analysis. The resulting four derivatization mixtures were subjected to LC–MS analysis with a Phenomenex Kinetex C18 reversed-phase column (2.6 μm, 100 × 4.6 mm) at a flow rate of 1.0 mL/min and with a linear gradient of H₂O (containing 0.1% formic acid) and MeOH (90:10 to 0:100 over 15 min, and a hold at 100% ACN for 5 min). The retention times and ESI data for t-FDLA derivatives of the hydrolysates and the standard amino acids are summarized in Tables S5 and S6 (Supporting Information).

**Determination of Proteinogenic Amino Acid Configurations.** t- and d-FDLA were synthesized as previously reported. The hydrolysate was mixed with 1 N NaHCO₃ (40 μL), and 35 μL of t-FDLA (10 mg/mL in acetone). Each solution was stirred at 45 °C for 1 h, cooled to room temperature, quenched with 1 N HCl (40 μL), and dried under vacuum. Similarly, the standard t- and d-amino acid were derivatized separately. The derivatives of the hydrolysate of compound 1 and the standard amino acids were subjected to LC–MS analysis with a Phenomenex Kinetex C18 reversed-phase column (2.6 μm, 100 × 4.6 mm) at a flow rate of 0.5 mL/min and with a linear gradient of H₂O (containing 0.1% formic acid) and MeOH (90:10 to 0:100 over 15 min, and a hold at 100% ACN for 5 min). The retention times and ESI data for t-FDLA derivatives of the hydrolysates and the standard amino acids are summarized in Tables S5 and S6 (Supporting Information).
500 MHz): δ 4.09 (1H, m), 3.99 (1H, m), 3.73 (3H, s), 3.69 (3H, s), 3.63 (1H, br), 2.25 (1H, m), 2.01 (1H, m), 1.64 (1H, br), 1.04 (3H, d, J = 7.0 Hz), 1.03 (3H, d, J = 7.0 Hz), 0.99 (3H, d, J = 7.0 Hz), 0.71 (3H, d, J = 7.0 Hz). 1H NMR data of (R)-1-(2RS)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)-2-methylpropan-1-ol (CDCl3, 500 MHz): δ 4.10 (1H, t, J = 3.5 Hz), 3.92 (1H, t, J = 3.5 Hz), 3.65 (3H, s), 3.64 (3H, s), 3.59 (1H, br), 2.20 (1H, m), 1.76 (1H, m), 1.51 (1H, br), 0.98 (3H, d, J = 7.0 Hz), 0.85 (3H, d, J = 7.0 Hz), 0.84 (3H, d, J = 7.0 Hz), 0.63 (3H, d, J = 6.9 Hz).

Organisms, Media, and Antibiotic of in Vivo Studies. Eudistamide B (2) was tested for antibacterial activity in vivo studies. One isolate of E. coli ATCC 29222 and S. aureus ATCC 25923, separately, was used for these studies. Organisms were grown, subcultured, and quantified using Mueller-Hinton broth (MHB) and agar (Difco Laboratories, Detroit, MI).

Animals. Six week-old, specific pathogen-free, female ICR/Swiss mice weighing 24–27 g were used for all studies (Harlan Sprague–Dawley, Indianapolis, IN). Animals were maintained in accordance with the American Association for Accreditation of Laboratory Animal Care (AAALAC) criteria. All animal studies were approved by the Animal Research Committees of the William S. Middleton Memorial VA Hospital and the University of Wisconsin.

Murine Thigh Infection Model. Mice were rendered neutropenic (neutrophils <100/mm3) by injecting cyclophosphamide (Mead VA Hospital and the University of Wisconsin. Animal Research Committees of the William S. Middleton Memorial VA Hospital and the University of Wisconsin. The authors declare no competing financial interest.


