# The role of D-*allo*-isoleucine in the deposition of the anti-*Leishmania* peptide bombinin H4 as revealed by $^{31}$P solid-state NMR, VCD spectroscopy, and MD simulation

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20

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**Abbreviations:** H2, bombinin H2; H4, bombinin H4; D-*allo*-Ile, D-*allo*-isoleucine; DOPC,1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPE,1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOPI, 1,2-dioleoyl-sn-glycero-3-phosphoinositol; DOPS, 1,2-dioleoyl-sn-glycero-3-phosphoserine; ERG, ergosterol; NMR, nuclear magnetic resonance; MAS, magic-angle spinning; DD, dipolar decoupling; VCD, vibrational circular dichroism; MD, molecular dynamics; DFT, density functional theory; MFP, magnetic field perturbation; IR, infrared; FTIR, Fourier-transform infrared; RMSD, root mean square deviation.

**Abstract**

Bombinin H4 is an antimicrobial peptide that was isolated from the toad *Bombina variegata*. Bombinin H family peptides are active against gram-positive, gram-negative bacteria, and fungi as well as the parasite *Leishmania*. Among them, bombinin H4 (H4), which contains D-*allo*-isoleucine (D-*allo*-Ile) as the second residue in its sequence, is the most active, and its L-isomer is bombinin H2 (H2). H4 has a significantly lower LC50 than H2 against *Leishmania*. However, the atomic-level mechanism of the membrane interaction and higher activity of H4 has not been clarified. In this work, we investigated the behavior of the conformations and interactions of H2 and H4 with the *Leishmania* membrane using $^{31}$P solid-state nuclear magnetic resonance (NMR), vibrational circular dichroism (VCD) spectroscopy, and molecular dynamics (MD) simulations. The generation of isotropic $^{31}$P NMR signals depending on the peptide concentration indicated the abilities of H2 and H4 to exert antimicrobial activity via membrane disruption. The VCD experiment and density functional theory calculation confirmed the different stability and conformations of the N-termini of H2 and H4. MD simulations revealed that the N-terminus of H4 is more stable than that of H2 in the membrane, in line with the VCD experiment data. VCD and MD analyses demonstrated that the first and residues, L-Ile and D-*allo*-Ile, respectively, in H4 tend to take a *cis* conformation. These residues function as an anchor and facilitate the easy winding of the helical conformation of H4 in the membrane. It may assist to quickly reach to the threshold concentration of H4 on the *Leishmania* membrane.

**Keywords:** *Leishmania* mimetic membrane, antimicrobial peptide, bombinin H4, D-*allo*-isoleucine, molecular dynamics, solid-state nuclear magnetic resonance

## 1. Introduction

Molecular chirality strongly affects the structure and function of D-amino acid-containing peptides and proteins. The octopine peptide, which contains D-amino acids, was first discovered and isolated from the muscle of the octopus in 1927 [1]. The peptide contains D-alanine and functions as a lactic acid in the organism [2]. Dermorphin, a stronger and more potent anesthetic D-amino acid-containing peptide, was isolated from the skin secretions of the tree frog *Phyllomedusa sauvagii* [3]. Dermorphin contains D-alanine encoded by the normal L-alanine GCG codon in the second position in its sequence. This finding infers that the peptide is modified post-translationally [4,5]. α-Crystallin is one of the major proteins in the human lens. The age-dependent isomerization of L-Asp residues to the D-form is closely related to cataract progression [6]. Generally, a D-amino acid that is specifically present in various peptides and proteins plays a crucial role in regulating and enhancing their functions [7,8,9].

Antimicrobial peptides have attracted great interest due to their strong antibiotic activity [10–12]. These peptides form an amphipathic helical structure in the membrane and destroy the microbial cell membrane via different mechanisms such as forming pores or channels (toroidal-pore and barrel-stave) and exerting detergent-like (carpet-like) activity [12]. Interactions between the peptides and membranes play important roles in the actions of antimicrobial peptides [13]. D-amino acid-containing antimicrobial peptides have been isolated from frog skin secretions [14,15]. The bombinin H peptide family was isolated from the skin secretions of *Bombina* species [16-18]. These peptides are active against various microorganisms including gram-

positive and gram-negative bacteria, fungi, and the parasite *Leishmania* [19–22]. The amphibian peptides include bombinin H2 (H2) and its diastereomer bombinin H4 (H4), both of which were isolated from the skin of the yellow-bellied toad *Bombina variegata* [23]. H4 contains D-*allo*-isoleucine (D-*allo*-Ile) at the second position in its sequence due to post-translational modification of L-Ile in H2, in which the chirality of α-carbon is stereochemically changed [24,25]. Both peptides have 20 amino acid residues, and three positive charges, including Lys18 and Lys19, are present in the N-terminus. Therefore, both peptides more strongly bind to the acidic phospholipids located on the membrane surface [26]. The sequences of H2 and H4 peptides are as follows:

Bombinin H2:

Ile-(L-Ile)-Gly-Pro-Val-Leu-Gly-Leu-Val-Gly-Ser-Ala-Leu-Gly-Gly-Leu-Leu-Lys-Lys-Ile-$NH_2$

Bombinin H4:

Ile-(D-*allo*-Ile)-Gly-Pro-Val-Leu-Gly-Leu-Val-Gly-Ser-Ala-Leu-Gly-Gly-Leu-Leu-Lys-Lys-Ile-$NH_2$

The infectious disease leishmaniasis has an estimated incidence of approximately 12 million cases globally [27]. H2 and H4 were previously assayed in *Leishmania* promastigotes and amastigotes [20]. Although the membrane of *Leishmania* is extremely resistant to mechanical stress and chemical or biological agents [28,29], experimental data indicate that H2 and H4 damage the membrane, resulting in the loss of intercellular materials including proteins. Moreover, H4 has remarkably higher bactericidal, fungicidal, and anti-*Leishmania* activity than H2, excluding activity against *Aeromonas hydrophila* Bo-3N [20,21,30]. Surface plasmon resonance and two-phase quantitative models (initial binding and insertion) confirmed that H4 has greater binding

affinity and hydrophobicity than H2 [16,20]. H4 has 5-fold stronger binding affinity for the model membrane than H2 [20,31]. Attenuated total reflectance–Fourier transform infrared (FTIR) spectroscopy and CD spectroscopy revealed that the D-amino group did not affect the α-helical structure of H4, and H2 more easily forms a β-sheet in *Leishmania* mimetic membranes [20]. The structures of H2 and H4, containing flexible and randomly ordered N-terminal conformations, were determined in a mimetic membrane environment via solution nuclear magnetic resonance (NMR) spectroscopy [32]. The N-terminus was demonstrated to form part of the interaction site between the helices [32]. The folding structure of H4 was a little loose compared to that of H2 from the circular dichroism measurements and molecular dynamics (MD) simulations in aqueous solution [33]. The MD analysis illustrated that D-*allo*-Ile reduces the intra-peptide interactions that affect peptide folding [33].

The inclusion of D-amino acids in peptides is a natural mechanism for modulating their antimicrobial activity. Although Mangoni *et al.* observed similar bacterial susceptibility to H2 and H4, one of the existing explanations of the higher antimicrobial activity of H4 is its resistance to enzymatic degradation and serum clearance because of the presence of D-*allo*-Ile [20,31]. Moreover, when the rotation of a D-amino acid-containing side chain is restricted and sandwiched by two neighboring L-residues, new membrane interaction activity that cannot be achieved with the all L-amino acid-containing sequence is observed [34]. Simmaco *et al*. considered that a specific arrangement of L- and D-amino acids could promote higher bioactivity and extra-functional properties [18]. Mangoni *et al*. also revealed that the stronger affinity of H4 is caused by initial binding opposed to an insertion, and both peptides similarly coincide with the peptides that act via the carpet mechanism [20]. Conversely, the

peptide-peptide association is a possible factor for the higher activity of H4 against *Leishmania*. Detailed information and atomic-level explanations regarding the interaction with the *Leishmania* membrane and the higher activity of H4 have not been elucidated.

Solid-state NMR spectroscopy has been extensively used to clarify the dynamic structures of various types of antimicrobial or antibiotic peptides in a membrane [35-39]. The first solid-state NMR structure of a channel dimer of gramicidin A with alternating D- and L-amino acid sequences was determined using $^{15}N$ NMR orientational constraints [40]. In addition, analyzing the oscillation of the $^{13}C$ chemical shift anisotropy of $^{13}C$-labeled peptides gave a dynamic structure of an antimicrobial peptide bound to a membrane [37,38]. MD simulations can investigate the dynamic behavior of antimicrobial peptides in the membrane environment and permit comparisons with experimental data [41]. In this work, we comparatively explored the dynamic structures of the peptide and membrane interactions of H2 and H4 on a *Leishmania* mimetic membrane using $^{31}P$ solid-state NMR, vibrational circular dichroism (VCD) spectroscopy, and MD simulations. In particular, VCD and MD simulations clarified the importance of the D-amino acid in the stronger antimicrobial activity of H4. The "carpet-like" antimicrobial mechanism of H2 and H4 was observed for the *Leishmania* mimetic membrane via $^{31}P$ solid-state NMR experiments. VCD and MD simulations agreed that the first and second residues (Ile and D-*allo*-Ile) of H4 tend to take a *cis*-like conformation. We introduce an atomic-level explanation of the higher binding affinity of H4 for the *Leishmania* mimetic membrane.

## 2. Materials & methods

### 2.1 Peptide synthesis

H2 and H4 were synthesized via microwave-assisted solid-phase peptide chemistry using an Initiator+ Alstra peptide synthesizer (Biotage). For $^{13}C$ solid-state NMR experiments, singly labeled [1-$^{13}C$]Leu6 H2 and H4 were also synthesized. These peptides, which exhibited different retention times, were purified using a reversed-phase HPLC system (Shimadzu) equipped with a Kinetex Axia C18 ODS column, and the purity of both peptides was >95%. We re-checked the diastereomeric separations of the purified peptides on a CHIRALPAK IF column (Daisel) (Fig. S1). H2 and H4 were also confirmed by observing that the mass number was [M+H] = 1917.25 m/z as determined via MALDI-TOF-MS (Bruker Daltonics).

### 2.2 NMR sample preparation and solid-state NMR experiments

We used a *Leishmania* mimetic membrane containing 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoinositol (DOPI), 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS), and ergosterol (ERG) at a molar ratio of 4:4:2:1:6. Although the molar ratio of only DOPC is slightly different for the NMR experiments, the lipid content is the same as that used by Mangoni and co-workers [20]. The mixture of lipids and ERG was dissolved in organic solvent (methanol/chloroform, 1:1 [v/v]) and subsequently evaporated to create a thin film. This film was carefully hydrated with Tris-NaCl buffer (pH 7.4), and then the lipids were incubated overnight at 40°C. The

peptide was dissolved in the suspension, and the peptide-lipid (P/L) mixture was incubated at 40°C for 2 days. In the $^{31}$P solid-state NMR experiments, the P/L molar ratio was adjusted to 1:20, 1:200, or 1:2000. We also prepared a *Staphylococcus aureus* mimetic membrane containing 1,2-dimyristoyl-sn-glycero-3-phosphorylcholine, 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol sodium salt, and cardiolipin at a molar ratio of 12:5:1.

All solid-state NMR experiments were performed on a Bruker Avance III 600 MHz spectrometer equipped with a double resonance standard-bore probe at the $^{1}$H, $^{13}$C, and $^{31}$P resonance frequencies of 600.15, 150.90, and 242.95 MHz, respectively. $^{13}$C and $^{31}$P NMR signals were measured with high-power proton dipolar decoupling (DD) under static and magic-angle spinning (MAS) conditions ($^{31}$P DD-static and $^{13}$C DD-MAS) at 40°C for fully hydrated samples. $^{13}$C and $^{31}$P chemical shift values were determine in reference to glycine powder at 176.03 ppm (TMS: 0.00 ppm) and 85% $H_3PO_4$ at 0.00 ppm, respectively. The NMR data were processed and analyzed using the Bruker TOPSPIN program. $^{31}$P NMR spectra were deconvoluted to estimate the relative isotropic components.

**2.3 VCD experiments**

Peptide samples for VCD experiments were prepared by mixing a peptide and KBr at a ratio of 1:100 and placing the mixture into a transparent 10-mm-diameter pellet. VCD and infrared (IR) signals were observed using a JASCO PRESTO-S-2016 VCD/LD spectrometer. The IR intensity was adjusted to approximately 0.5. The sample cell was rotated along the direction of monitoring at 0 and 45° to confirm the reliability.

The signals were accumulated for 10,000 scans for each sample. The IR and VCD spectra of these complexes were theoretically calculated using the Gaussian 09 program (E.01). The VCD intensities were determined using the vibrational rotational strength and magnetic dipole moments, which were calculated by the magnetic field perturbation theory formulated using magnetic field gauge-invariant atomic orbitals. The calculated intensities were converted to Lorentzian bands with a 4 $cm^{-1}$ half-width at half-height. Geometric optimization was performed at the density functional theory (DFT) level (B3LYP functional) with 6-31G(d,p) basis sets.

**2.4 MD simulations**

MD simulations were performed for H2 and H4 in the *Leishmania* mimetic membrane. All simulations were calculated with GROMACS 5.1 [42] using CHARMM36 force fields [43]. As initial structures of the simulations, three different positions of the peptides on the membrane surface were constructed for both H2 and H4 (A, B, and C types in Fig. 3) using the CHARMM-GUI membrane builder and *editconf* tool of the GROMACS package. The coordinates of H2 and H4 were taken from PDB entries 2ap7 and 2ap8, respectively [32]. Each of these PDB entries contains 20 conformations that were derived via solution NMR. The OLDERADO database was used to analyze the conformations, illustrating that the most representative models for H2 and H4 are the $12^{th}$ and $6^{th}$ models, respectively [44]. These models were used in this study. The structures with protonated N-termini ($NH_3^+$) and amidated C-termini ($NH_2$) were considered in this work as the neutral pH condition. The net charges of the structures are equal to +3 for both peptides. All systems were solvated using the water model of TIP3P [45] with 100 mM NaCl. The *Leishmania* mimetic membrane consists

of DOPE, DOPC, DOPI, DOPS, and ERGs (the corresponding molar ratio was 4:4:2:1:6). As the structure and charmm topology file of D-*allo*-Ile and DOPI were not available in the membrane builder, we manually modified those of isoleucine and POPI, respectively. Detailed information about the simulation system, including the numbers of lipid, ion, and water molecules, is shown in Table 1. The periodic boundary condition was applied in all directions. After the standard minimization and equilibration procedure of the CHARMM-GUI membrane builder [46] at 313 K (a total of 1.1 ns of equilibration with a 1-fs step), 1 μs of simulations was performed without any restrains for all systems using a constant number of atoms, pressure, and temperature ensemble. The time step of the simulation was 2 fs, and the neighbor list was updated in every 20 steps [47]. Data were saved every 2 ps. Bond lengths involving hydrogen atoms were fixed using the LINCS algorithm [48]. Lennard-Jones interactions were switched off at 10–12 Å with a force-based switching function [49]. The long-range electrostatic interactions were calculated using the particle-mesh Ewald method [50,51]. The system was coupled to a temperature bath at 313 K with a coupling constant of 1.0 ps using a Nose-Hoover thermostat [52,53]. A semi-isotropical Parrinello-Rahman barostat maintained the pressure at 1 bar with $\tau_P$ = 5.0 ps and compressibility of $4.5 \times 10^{-5}$ $bar^{-1}$ [54,55]. All analyses, excluding the secondary structure assignment of the DSSP program [56], were performed using standard tools of the GROMACS package. The structural visualizations were displayed using visual molecular dynamics (VMD) [57] and Chimera software [58]. Xmgrace was used for the graphic representation of MD simulations [59].

## 3. Results and Discussion

### *3.1 Leishmania* mimetic membrane disruption

Figure 1 shows the $^{31}$P static NMR spectra of the *Leishmania* mimetic lipid bilayers at 40°C. First, a powder pattern of the gel phase of the membrane was obtained (Fig. 1A). At a P/L ratio of 1:20, a strong isotropic NMR signal appeared at 0.07 ppm, indicating that H2 or H4 induces fully disruption of the membrane into small lipid particles [36,39]. Therefore, the spectral area of the relative isotropic component at a P/L ratio of 1:20 was estimated to be approximately 100% compared with the powder pattern signal. At a P/L ratio of 1:200, H2 and H4 disrupted the membrane with a spectral area of around 40%. At a lower peptide concentration (P/L ratio: 1:2000), a similar powder pattern as observed in the absence of a peptide was obtained. The $^{31}$P NMR spectra of the *Staphylococcus aureus* mimetic membrane revealed axial symmetric powder patterns (Fig. S2). $^{31}$P chemical shift anisotropy of the membrane with H2 or H4 was reduced compared with that for only *S. aureus* mimetic membranes, but the isotropic signal near 0 ppm was not observed even at high peptide concentrations (P/L ratio = 1:20), in contrast to the findings for the *Leishmania* mimetic membrane system (Fig. 1). Our $^{31}$P solid-state NMR results indicate that the bombinin peptides self-associate on the *Leishmania* membrane through a specific P/L interaction and then induce detergent-like solubilization upon reaching a high peptide concentration (carpet mechanism). However, the changes of the $^{31}$P NMR spectral patterns for the two membrane systems between H2 and H4 were extremely similar. In the $^{13}$C DD-MAS NMR spectra of [1-$^{13}$C]Leu6 of H2 and H4 in the *Leishmania* membrane, the NMR signals of both H2 and H4 appeared at approximately 177.15 ppm, indicating that the

peptides formed α-helical structures in the membrane (Fig. S3) [60]. D-*allo*-Ile in H4 may cause more fluctuation on the *Leishmania* membrane, as the only difference was the higher $^{13}C$ signal intensity.

Although previous researchers found that H4 exhibits stronger activity against *Leishmania* than H2, our solid-state NMR data indicate that the peptides possess similar membrane disruption activities. A possible explanation for this finding is that H2 as well as H4 may be fully reacted with the membrane at 40 ºC due to the long duration from sample preparation to the accumulation of NMR signals. Actually, Mangoni et al. reported that *Leishmania* donovani protozoa incubated for 1 hour with peptides were killed around 80% at 32°C [20].

**3.2 VCD analysis of the N-terminal configurations**

VCD is a powerful tool for determining the absolute configuration of chiral molecules [61,62]. We observed the solid-state VCD spectra of L-Ile and its related amino acids (L-*allo*-Ile, D-*allo*-Ile, and D-Ile) prior to the current experiments. Significant positive/negative signs of the VCD peak gave insights into the interplay between the two chiral carbon centers of Ile [63]. Figure 2A shows the observed solid-state VCD (upper) and IR (lower) spectra of H2 and H4 in KBr. The blue line is the baseline for only KBr. As expected, several VCD vibrations of a peptide containing 20 amino acids gave a spectral complex that made it difficult to recognize vibrations. Several VCD peaks were observed in the wave number region of 1400–1800 $cm^{-1}$. Fortunately, the H2 and H4 samples gave VCD peaks with opposite signs, especially around 1650 $cm^{-1}$, under nearly the same IR intensity. The solution NMR structures of

291 H2 and H4 were similar excluding the N-terminus [32]. This finding indicated that this
292 vibration is attributed to the chiral difference of the N-terminal structures. Therefore, the
293 calculated VCD is based on the four N-terminal residues (H2, (L-Ile)-(L-Ile)-Gly-(L-Ile);
294 H4, (L-Ile)-(D-*allo*-Ile)-Gly-(L-Ile)) (Fig. 2B). The IR and VCD spectra were calculated
295 for the optimized structures. VCD peaks 1–3 in the calculated spectra agree with the
296 observed peaks. The opposite signs near 1530 $cm^{-1}$, which originated from NH bending
297 of the second and third residues, are unclear in the observed VCD peaks at 1420 $cm^{-1}$
298 because of the similarity with the baseline data. The DFT calculations revealed that the
299 most stable conformations of the first two side chains are the *trans* form for H2 and the
300 *cis* form for H4 (Fig. 2C and D). Because the first two side chains of H4 are arranged on
301 one side to the peptide backbone, H4 has a larger hydrophobic component on the N-
302 terminal side. Indeed, in our HPLC analysis with ODS and CHILARPAK IF columns,
303 the retention time of H4 was prolonged by a few minutes (Fig. S1).
304
305 **3.3 MD simulations of H2 and H4**
306 MD simulations were performed to investigate the structure, dynamics, and
307 interactions of H2 and H4 with the *Leishmania* mimetic membrane systems. As the
308 initial structures, H2 and H4 were built to take an α-helical conformation inside the
309 membrane bilayer [32]. They were placed at three different initial positions in the
310 membrane-water systems as shown in Fig. 3. The first one is type A, in which H2 and
311 H4 are placed in the water region above the membrane surface. The second is type B, in
312 which the peptides are placed in a pseudo-transmembrane position. In this model, Lys18
313 and Lys19 are placed at the interface between the membrane surface and water. The last
314 one is type C, in which the peptides are placed in a transmembrane position. Figure 3

315 shows snapshots of the dynamic behaviors during the simulation of the peptides in the
316 systems. In the type A simulation, the α-helical structures of both H2 and H4 were
317 unwound, resulting in some distorted conformations in the early stage of the simulation.
318 These results indicate that the α-helix conformations of bombinin peptides are unstable,
319 and a random coil structure is taken in a water environment. After 30 ns of simulation
320 time, the N-terminal side of H4 inserted into membrane surface. Similarly, the same side
321 of H2 inserted into the membrane surface at approximately 635 ns. Conversely, the α-
322 helical conformation was maintained throughout the entire simulation period for both
323 types B and C. These results indicated that bombinin H peptides can take α-helical
324 conformations in membrane environments despite taking random coil conformations in
325 water. Based on the A type simulation, we could investigate the initial insertion of the
326 peptides into the membrane. However, the process of helix formation after insertion into
327 the membrane could not be observed in this work. It should be a long process to observe
328 the transition of the peptide conformation from a coil to an α-helix in the membrane,
329 which could not be observed in this work. However, the snapshot at 635 ns for H4 in the
330 A type simulation appeared to illustrate the beginning stage of helix formation of the
331 peptide in the membrane, the structure of which is shown in Fig. 8B in more detail and
332 discussed in a later section.

333 In the B simulations, both H2 and H4 displayed similar behavior. They gradually
334 entered the membrane center by keeping their α-helix conformations and finally took
335 transmembrane orientations. The final orientations of the B type simulation are similar
336 to those of the C type simulation. This result clearly demonstrated that both H2 and H4
337 finally took a stable transmembrane orientation once they inserted into the membrane.

H2 and H4 form stable α-helical structures with similar tilt angles in the membrane. The formation of the α-helix conformation was confirmed by the DSSP analysis, and the results are shown in Fig. S5. The average values of the tilt angles in the last 500 ns were also analyzed and presented in Table S1. These similarities in the membrane indicate that once H2 and H4 are fully inserted into the membrane, they possess similar anti-*Leishmania* activity, as shown in Fig. 1 using $^{31}$P solid-state NMR spectra. The spectra might have been measured in the fully inserted stage for both H2 and H4 because of the long duration of the experimental procedures. The secondary structures of inserted H2 and H4 are in good agreement with the results of $^{13}$C solid-state NMR (Fig. S3), CD, and FTIR studies, as they predominantly formed α-helical structures in the membrane [20,21,33].

**3.4 Dynamic behavior of H2 and H4**

To investigate the difference of the insertion processes between H2 and H4 in more detail, the behavior of the dynamics of the peptide was analyzed. The conformation dynamics of the peptide backbone was calculated using root mean square deviations (RMSDs) from their initial conformations as a function of time, as shown in Fig. 4. In the water region (type A), both peptides exhibited similarly large fluctuations in the RMSD measurements (black and red lines). Conversely, some small differences in dynamic behavior between H2 and H4 could be observed in the types B and C simulations. In both types B and C membrane systems, the RMSDs of H2 (green and yellow lines) were slightly larger than those of H4 (blue and purple lines). In Fig. 4, the structure of H2 (green line) exhibited greater fluctuations than that of H4 (blue line) during the type B simulation. The greater fluctuation of H2 was also apparent in the

type C simulation. This finding indicates that the dynamic behavior of H4 is more stable than that of H2 in membrane environments. To investigate the molecular motion of each residue in H2 and H4, the root mean square fluctuation of the backbone atoms of each residue of these peptides was analyzed, as shown in Fig. 5 according to the residue numbers. The data illustrate that the residues on the N-terminal side are more flexible than those in other parts in the membrane environment. This result agrees with the solution NMR structures of H2 and H4 [32]. Moreover, the residues on the N-terminal side of H2 (green and yellow lines) are more flexible than those in H4 (blue and purple lines) in the membrane. Figures 4 and 5 indicate that H4 has higher structural stability than H2 in the membrane. As the only difference between these peptides is the different chirality of the second residue, the different behavior should be attributable to D-*allo*-Ile. In the following section, we analyzed the interaction between the amino acid residues on the N-terminal side and the lipid molecules.

### 3.5 The deposition behaviors of H2 and H4 on the *Leishmania* mimetic membrane

Type A (A1 and A2) simulations allow us to consider the initial insertion, deposition, and interaction behaviors of H2 and H4 with the *Leishmania* mimetic membrane. Figure 6 shows the Z coordinates of the first two residues on the N-terminal side of H2 and H4 during the 1000-ns simulation time. The position of the membrane surface was calculated using the average value of the Z coordinates of the phosphorus atoms of lipids in the upper leaf of the membrane. In both the A1 and A2 simulations, the first two residues of H2 and H4 inserted into the membrane early in the simulation. The Z coordinate of residue 2 (Ile2, green line) of H2 was lower than that of the membrane surface during all times after 100 ns (Fig. 6A). That is, the Ile2 residue can

386 be considered to interact with and insert into the membrane surface after 100 ns of
387 simulation. However, Ile1 (red line) of H2 had not yet been fully inserted into the
388 membrane even after 100 ns of simulation. The residue finally fully inserted and
389 interacted with membrane after 800 ns. Conversely, Ile1 (red line) and D-*allo*-Ile2 (blue
390 line) of H4 both rapidly inserted into the membrane surface within 30 ns in the A2
391 simulation (Fig. 6B).

392 To investigate the difference of these interaction behaviors in more detail, the
393 conformations of the first two residues of H2 and H4 were surveyed by analyzing the
394 pseudo-dihedral angle (*cis*/*trans* form) between these two side chains. The definition of
395 the pseudo-dihedral angle is shown in Fig. S4, and the results are shown in Fig. 7 as a
396 function of time. The analysis demonstrated that Ile1 and D-*allo*-Ile2 in H4 formed a
397 *cis*-like conformation immediately after the start of the simulation and maintained this
398 conformation throughout the simulation. That is, Ile1 and D-*allo*-Ile2 in H4 formed a
399 *cis*-like conformation in water environments and after insertion into the membrane.
400 Meanwhile, Ile1 and Ile2 in H2 took a *trans*-like conformation until 800 ns of
401 simulation time. However, a *cis*-like conformation was taken after 800 ns. The timing of
402 this conformational change of H2 corresponds to full insertion of the two residues of H2
403 into the inside of the membrane. The VCD spectra discussed in the previous section also
404 illustrated that the most stable conformation of the first two side chains of H2 is *trans*,
405 and versus *cis* for H4. These results indicated that the sequence of L-Ile and D-*allo*-Ile
406 favors the formation of *cis*-like conformations compared to L-Ile and L-Ile, and this
407 sequence enabled interaction with hydrophobic parts in the membrane more easily than
408 observed in H2. The values of the proportion of *cis*-like conformations of H2 and H4
409 during the A1 and A2 simulations were calculated, as shown in Table 2. In this table, the

simulation that was divided into three sections: 0–200, 200–800, and 800–1000 ns. The data illustrated that both H2 and H4 finally take *cis*-like conformations in the membrane environment. However, H4 can take *cis*-like conformations early in the simulation. The adjacent L-Ile and D-*allo*-Ile with *cis*-like conformations would facilitate the rapid interaction and deposition on the *Leishmania* mimetic membrane. To elucidate the role of the *cis* conformation in the interaction with the membrane, snapshots of the interaction behaviors are shown in Fig. 8. Figure 8A and 8B demonstrates the mechanism by which H2 and H4 insert into the membrane for the first time during the simulations. It can be observed that the side chains of L-Ile and D-*allo*-Ile of H4 entered the membrane, functioning as an anchor to interact with the membrane surface using two hydrophobic interaction sites with *cis* conformations (Fig. 8B). In H2, although Ile2 entered the membrane, Ile1 oriented toward the upper side of the membrane surface. This would weaken the interaction of the residue with the membrane. These results are in good agreement with the surface plasmon resonance measurement and two-state reaction model, which indicated that H4 has greater binding affinity than H2 [20]. Regarding H2, it can be observed that the hydrophobic side chains of other parts of the peptide residues (Leu8 and Val9 in the case of Fig. 8A) interact with the membrane surface. By considering the slow process of insertion of H2 into the membrane, the interactions of these residues with the membrane may not accelerate the insertion but block the specific suitable orientation for the insertion of bombinin. In fact, the snapshots of H2 and H4, which were deeply inserted into the membrane during the simulations, are shown in Fig. 8C and 8D, respectively. The conformation of the inserted part of H2 illustrated that some hydrophobic side chains of the peptide orient toward the inside of the membrane. However, those interactions appear to distort the

conformation of H2, which would prevent quick insertion of the peptide into the inside of the membrane. Conversely, a part of the inserted residues in the middle part of H4 (residues 8–13) was demonstrated to rewind the helix conformation. The well-mannered insertion from the N-terminal side using the specific interaction with the *cis*-like conformation of L-Ile and D-*allo*-Ile would be considered to both function as an anchor toward the membrane surface interaction and facilitate the easy winding of the helical conformation after insertion into the membrane. Our A type simulations demonstrated that both H2 and H4 can deposit on the membrane surface. After their concentration reached the threshold values, they act via a carpet-like mechanism. From our suggested role of D-*allo*-Ile, the concentration of H4 on the membrane surface is accelerated via quicker insertion of its first two residues. Simmaco *et al*. considered that a specific arrangement of L- and D-amino acids in H4 might generate a new structural and functional element that cannot be formed in all L-isomers [18]. This finding is explained by our suggested role of D-*allo*-Ile in the deposition of bombinin H4.

The interaction between the two residues on the N-terminal side of H2 and H4 and the membrane was investigated, and the results are shown in Fig. 9. The electrostatic and van der Waals interaction energies were evaluated separately and shown in panels (A) and (B), respectively. Regarding the electrostatic interaction energy, there was no large difference between H2 and H4. However, the van der Waals interaction energy between the first two residues of H4 and the membrane was larger than that for H2. This indicated that the L-Ile and D-*allo*-Ile residues of H4 favor interactions with the membrane surface compared to H2. Table 3 shows the contribution of the energies from the different types of membrane molecules. The membrane molecules of DOPC, DOPI, and EGR exhibited similar contributions to the interaction

458 energies with the two residues regarding the electrostatic and van der Waals interaction
459 terms for both H2 and H4. However, DOPE and DOPS displayed different contributions
460 to the interaction energy. For both membrane molecules, the two residues of H4
461 exhibited stronger interactions than those of H2. The most comparable example is the
462 anti-*Leishmania* activity of temporin SHa and its analog [$K^3$]SHa peptides, both potent.
463 Because temporin [$K^3$]SHa is possible to initially stronger interact with membrane due
464 to presence of third lysine residue, it displays more efficient and faster killing ability
465 than SHa [64]. Moreover, H4 showed slightly faster reached the plateau of
466 depolarization compared to H2 on *Leishmania* promastigotes [20]. Therefore, the initial
467 membrane interaction kinetics of the peptides may be coupled with the rate of
468 expression.

469 In addition, peptide-peptide association is one of the possible causes of the
470 higher activity of H4. This was not examined in this work because of the lack of
471 experimental data regarding the peptide-peptide interactions and associations. Although
472 our solid-state NMR experiments focused on the difference between the association of
473 H2 and H4, we could not clarify the contribution of peptide-peptide association. Future
474 works will thoroughly investigate this matter via combinations of experimental and
475 theoretical methods.

476

477 **4. Conclusions**

478 The "carpet-like" mechanism of H2 and H4 against *Leishmania* mimetic
479 membrane was observed using $^{31}$P solid-state NMR. VCD and MD analyses agree that
480 that the N-termini of the peptides exhibit different structural and functional features in

the membrane. Membrane interactions stabilize the N-terminus of H4, which is more stable than that of H2 in the *Leishmania* mimetic membrane. H2 and H4 exhibit different timings of the initial binding with the membrane. We suggest that the specific arrangement of L-Ile and D-*allo*-Ile of H4 facilitates rapid formation of a *cis*-like conformation, and this confirmation plays an anchoring role in the deposition and penetration of H4. Additionally, the ordering insertion helped the peptide to wind the helix easily inside the membrane. Contrarily, H2 gradually attaches to the membrane surface using several hydrophobic and charged amino acids. The rapid insertion of these residues of H4 supports the rapid achievement of the threshold concentration. Consequently, H4 exerts its effects more rapidly than H2. However, once H2 and H4 are sufficiently inserted into the membrane and they form an α-helix structure, they have similar ability to destroy *Leishmania* mimetic membranes according to the dynamics behavior of the simulation in the membrane.

## Conflicts of Interest

There are no conflicts of interest to declare.

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