

# Conformational Changes in Alamethicin Associated with Substitution of Its $\alpha$ -Methylalanines with Leucines: A FTIR Spectroscopic Analysis and Correlation with Channel Kinetics

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**ABSTRACT** Alamethicin, a 20 residue-long peptaibol remains a favorite high voltage-dependent channel-forming peptide. However, the structural significance of its abundant noncoded residues ( $\alpha$ -methylalanine or Aib) for its ion channel activity remains unknown, although a previous study showed that replacement of all Aib residues with leucines preserved the essential channel behavior except for much faster single-channel events. To correlate these functional properties with structural data, here we compare the secondary structures of an alamethicin derivative where all the eight Aibs were replaced by leucines and the native alamethicin. Fourier transform infrared (FTIR) spectra of these peptides were recorded in methanol and in aqueous phospholipid membranes. Results obtained show a significant conformational change in alamethicin upon substitution of its Aib residues with Leu. The amide I band occurs at a lower frequency for the Leu-derivative indicating that its  $\alpha$ -helices are involved in stronger hydrogen-bonding. In addition, the structure of the Leu-derivative is quite sensitive to membrane fluidity changes. The amide I band shifts to higher frequencies when the lipids are in the fluid phase. This indicates either a decreased solvation due to a more complete peptide insertion or a peptide stretching to match the full thickness of the bilayer. These results contribute to explain the fast single-channel kinetics displayed by the Leu-derivative.

## INTRODUCTION

The peptaibol alamethicin, just long enough to span a lipid bilayer, introduced 30 years ago as a model for ion channel protein, continues to be intensively studied (for review, see Woolley and Wallace, 1992; Sansom, 1993; Cafiso, 1994; Bechinger, 1997; Duclouhier and Wroblewski, 2001). Alamethicin is a 20 residue-long natural polypeptide endowed with antibiotic activity, extracted from the fungus *Trichoderma viride* as a mixture of related compounds (Martin and Williams, 1976) of which the main component has the sequence shown in Fig. 1.

Alamethicin forms voltage-gated ion channels in planar lipid bilayers (Boheim, 1974; Eisenberg et al., 1973) and in cell membranes (Sackmann and Boheim, 1979), as well as inducing membrane excitability (Mueller and Rudin, 1968; Duclouhier and Spach, 2001). Alamethicin has recently been used in a tethered bilayer sensor with the potential application in drug discovery processes (Yin et al., 2003).

In addition to voltage-driven transmembrane incorporation of preformed interfacial aggregates (Schwarz et al., 1987; Vodyanoy et al., 1988), two main kinds of mechanisms have been proposed for the way in which this channel-forming peptide operates: 1), through conformational changes, and 2), the helix dipole models. For instance, Hall et al. (1984) proposed a conformational transition between a bent structure around Pro<sup>14</sup> with a C-terminal  $\beta$ -structure and a N-terminal  $\alpha$ -helix, and an all linear helix

as the basic gating event, broadly in line with Fox and Richards' (1982) hypothesis based on crystal structure. In models stressing the role of the helix dipole moment, some of the inserted helical monomers flip across the membrane dielectric so that transmembrane antiparallel bundles turn parallel (Menestrina et al., 1986). Despite existing evidence from the literature that alamethicin monomers adopts a predominantly  $\alpha$ -helical secondary structure in membranes (Esposito et al., 1987; Cascio and Wallace, 1988; Haris and Chapman, 1988; Bak et al., 2001), the structure of the helical bundle in the lipid bilayer is not known in detail, especially as regards its open state. However, some modeling and simulations studies offer interesting clues regarding membrane insertion and bundle assembly (e.g., Tieleman et al., 1999).

It has been argued that alamethicin may not constitute an appropriate model peptide for membrane proteins because of its high content of Aibs, uncoded amino acids. However the fact that it is a hydrophobic peptide that is predominantly  $\alpha$ -helical and whose crystal structure and solution structure are known makes it a useful peptide for understanding membrane protein folding and aspects of ion channel function. As it would be useful to deal with an analog of alamethicin that did not contain any of the Aib residues, thus removing the ambiguity of a partial  $3_{10}$ -helical character in alamethicin, a synthetic analog in which all the eight Aibs were replaced by leucine (designated alm-dUL, hereafter; see the amino-acid sequence in Fig. 1) was prepared. Its secondary structure is characterized here and compared to the native alamethicin using Fourier transform infrared (FTIR) spectroscopy to explain the functional modulation brought about by the Aib-Leu substitution.

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**Alamethicin:**

Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-PheOl

**Alm-dUL:**

Ac-Leu-Pro-Leu-Ala-Leu-Ala-Gln-Leu-Val-Leu-Gly-Leu-Leu-Pro-Val-Leu-Leu-Glu-Gln-PheOl

FIGURE 1 Amino acid sequences comparison of alamethicin and alm-dUL. Aib is  $\alpha$ -aminoisobutyric acid or  $\alpha$ -methylalanine, and Pheol is the amino-alcohol derivative of phenylalanine.

**MATERIALS AND METHODS****Materials**

Alamethicin was purchased from Sigma Chemical (St. Louis, MO; Product No. U-22324). Solvents were HPLC grade and the lipid L- $\alpha$ -DMPC (dimyristoyl-phosphatidylcholine) used in the FTIR study was purchased from Sigma Chemical. Lipids used for the single-channel conductance assay were palmitoyl-oleoyl phosphatidylcholine (POPC) and dioleoyl-phosphatidylethanolamine (DOPE), both from Avanti Polar Lipids (Birmingham, AL).

Purification of alamethicin and synthesis/purification of the alm-dUL analog.

The two main forms of alamethicin differ by the identity of residue at position 18: Glu or Gln for analogs referred as Rf<sub>30</sub> and Rf<sub>50</sub>, respectively, the charged form being more abundant. In addition, minor fractions have an alanine in place of  $\alpha$ -aminoisobutyric acid (differing by a CH<sub>2</sub>) at position 6. The two main alamethicin forms used here were separated using reversed phase HPLC with a Brownlee Aquapore (Santa Clara, CA) RP-300 C18 column, and the analysis was performed in a similar manner to that described by Gisin et al. (1977). As the FTIR spectra of the two forms of alamethicin were found to be identical, most of the measurements were made on the mixture as purchased from Sigma. As for the Leu-derivative, Alm-dUL, it was synthesized on solid-phase resin and HPLC-purified as previously described (Molle et al., 1988).

**Single-channel conductance assay in planar lipid bilayers**

For comparing the single-channel activity displayed by native alamethicin and Alm-dUL, bilayers were formed at the tip of patch-clamp pipettes as already described (Coronado and Latorre, 1983). The same standard conditions (e.g., 1 M KCl) and lipid mixture (POPC/DOPE; 7:3) already used before in most of our comparative conductance assays of a set of alamethicin derivatives (e.g., Molle et al., 1996) were used here. With other workers in the field, we found that this lipid mixture represents a good compromise for bilayer stability, ease of peptide insertion, and channel behavior. Indeed, correlated with an increased tendency for nonlamellar lipid structure or curvature strain, the incorporation of DOPE in planar bilayers favors more open substates and longer open times, i.e., some channel stabilization as shown by Keller et al. (1993).

**Samples preparation and Fourier transform infrared spectroscopy**

Spectra of the native alamethicin and of alm-dUL in methanol were obtained with 1–2 mg of the polypeptide dissolved in 100  $\mu$ l of the solvent. Spectra were recorded on a 1750 Perkin-Elmer (Wellesley, MA) FTIR spectrometer at 15°C and 25°C by signal averaging 64 scans with a resolution of 4 cm<sup>-1</sup>.

Native and synthetic alamethicin in lipid dispersions were prepared as follows. Required amounts of alamethicin and DMPC were dissolved in chloroform to achieve a peptide/lipid molar ratio of 0.05–0.15. After solvent evaporation under nitrogen, the samples were dried under vacuum. The required volume of H<sub>2</sub>O or <sup>2</sup>H<sub>2</sub>O was added to each of the dried samples, before mixing with a vortex and then incubated above the transition temperature of DMPC ( $T_i = 23^\circ\text{C}$ ) for 3–4 h. This incubation step was omitted for some samples in <sup>2</sup>H<sub>2</sub>O so that the hydrogen-deuterium exchange of the amide protons in the first several hours can be monitored. The concentration of alamethicin used in these measurements was ~10 mg/ml. Infrared spectra were recorded at 1°C, 15°C, 20°C, and 30°C by signal averaging 400 scans with a resolution of 4 cm<sup>-1</sup>. Further details about the methods of recording and analyzing FTIR spectra are described in our earlier publications (Haris et al., 1986, 1994; Haris and Chapman, 1988).

**RESULTS****Comparison of single-channel activity of native alamethicin and its Leu-substituted derivative in planar lipid bilayers**

As previously reported (Molle et al., 1988, 1996), all macroscopic and single-channel conductance data displayed by alm-dUL in planar lipid bilayers, as compared to native alamethicin, point to a very similar behavior as regards voltage- and concentration-dependences, and single-channel amplitudes. The only parameter that is significantly altered is the kinetics of single-channel substates as confirmed by the experiment shown in Fig. 2, performed for the purpose of

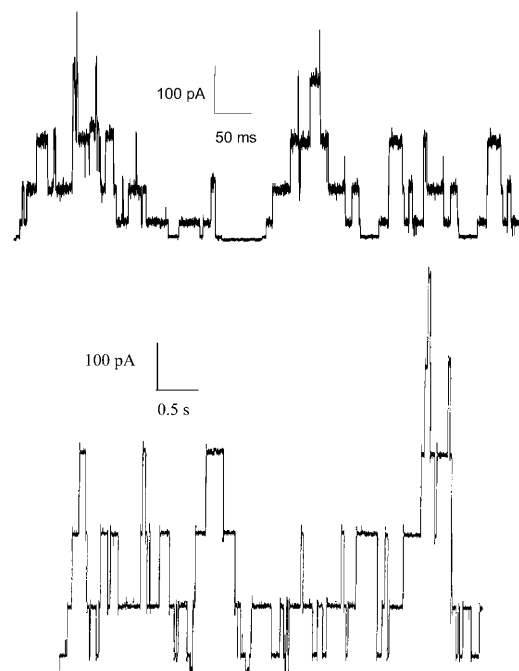


FIGURE 2 Comparison of single-channel current fluctuations induced by alm-dUL (top trace) and alamethicin (bottom trace) in POPC/DOPE (7:3) planar lipid bilayers in 1 M KCl solution and at the tip of patch-clamp pipettes. Bessel low-pass filter was set at 10 kHz and data were digitized at 30 kHz. Aqueous concentrations of alm-dUL and alamethicin were  $2 \times 10^{-9}$  M and  $5 \times 10^{-9}$  M, respectively, and the applied voltages were 150 mV (alm-dUL) and 120 mV (alamethicin). Temperature = 8°C.

this study: alm-dUL induces channels whose open lifetimes are about one order of magnitude faster than with native alamethicin (Fig. 2).

### FTIR spectra of native alamethicin and alm-dUL in methanol

Fig. 3 compares the FTIR spectra of native alamethicin with that of its Leu-derivative (alm-dUL), both in methanol solution. It can be seen that they are similar in the amide I and amide II regions. However, some differences are also apparent. For example, the amide I band for alm-dUL is more symmetrical compared to native alamethicin, and its peak occurs at a lower ( $\sim 3 \text{ cm}^{-1}$ ) than for alamethicin. On the other hand, the amide II band occurs at a lower frequency by  $1 \text{ cm}^{-1}$  for the native alamethicin. In addition, absorbances in the  $1675 \text{ cm}^{-1}$ ,  $1639 \text{ cm}^{-1}$ , and  $1621 \text{ cm}^{-1}$  regions are significantly lower for alm-dUL.

### Native alamethicin and alm-dUL in aqueous lipid dispersions

In general, the spectrum of alamethicin in aqueous lipid dispersion (Haris and Chapman, 1988) is similar to FTIR spectra of alamethicin recorded in methanol (Fig. 3). In that earlier study, FTIR spectra of native alamethicin in aqueous dispersions of DMPC, recorded above and below the lipid transition temperature, were shown to be virtually identical (Haris and Chapman, 1988).

The FTIR spectra of alm-dUL in lipid dispersion are similar to the spectra of native alamethicin also recorded in aqueous lipid dispersion. However, as for the samples in methanol (see Fig. 3), the main amide I component for alm-dUL occurs at a lower ( $2\text{--}4 \text{ cm}^{-1}$ ) frequency than for native alamethicin. This clearly indicates a change in the hydrogen-

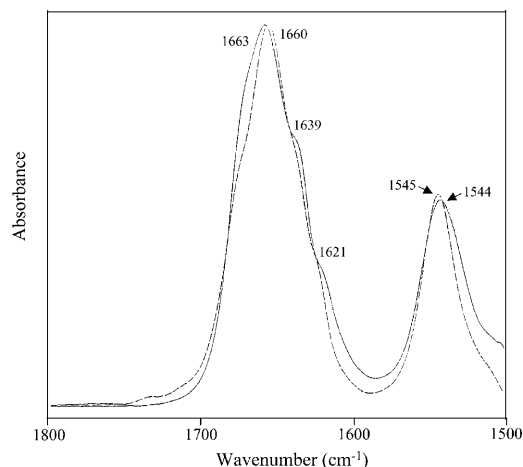


FIGURE 3 A comparison of the FTIR absorbance spectra of native alamethicin (continuous line) and alm-dUL (broken line). Both spectra were obtained for samples dissolved in methanol.

bonding pattern within the amide groups (Alvarez et al., 1987) of alm-dUL upon substitution of Aib residues with Leu.

Fig. 4 shows the second-derivative spectra of alm-dUL in aqueous lipid dispersion above ( $30^\circ\text{C}$ ) and below ( $15^\circ\text{C}$ ) the lipid phase transition. It can be seen that the main amide I component is at  $1657 \text{ cm}^{-1}$  below the lipid phase transition and this shifts to  $1661 \text{ cm}^{-1}$  when the temperature is raised above  $T_t$ . As the spectrum of this sample recorded at  $1^\circ\text{C}$  was found to be virtually identical to that recorded at  $15^\circ\text{C}$ , the observed shift above the lipid phase transition is not due to a direct effect of temperature on the polypeptide structure. It is also important to note that the amide II peak shifts from  $1548 \text{ cm}^{-1}$  to  $1546 \text{ cm}^{-1}$  on raising the temperature above the lipid phase transition. The simultaneous shift of the amide I and amide II band toward higher and lower frequency, respectively, suggests an increased hydrogen-bonding within the peptide groups (Alvarez et al., 1987).

### Native alamethicin and alm-dUL in lipid dispersions in deuterated water

The second-derivative spectrum of alamethicin in aqueous deuterated DMPC, recorded at  $15^\circ\text{C}$ , is presented in Fig. 5 A. This reveals a number of amide components. The main amide I component is at  $1659 \text{ cm}^{-1}$ , and a moderately strong component is observed near  $1634 \text{ cm}^{-1}$ . Weaker components are also observed near  $1618 \text{ cm}^{-1}$ ,  $1673 \text{ cm}^{-1}$ , and  $1698 \text{ cm}^{-1}$ . The other bands also shift toward lower frequency with the exception of the  $1617\text{-cm}^{-1}$  component. In the amide II region, the main component can be seen near  $1548 \text{ cm}^{-1}$ , and a weaker component occurs at  $1526 \text{ cm}^{-1}$ . FTIR spectra of this alamethicin sample in  $^2\text{H}_2\text{O}$  lipid

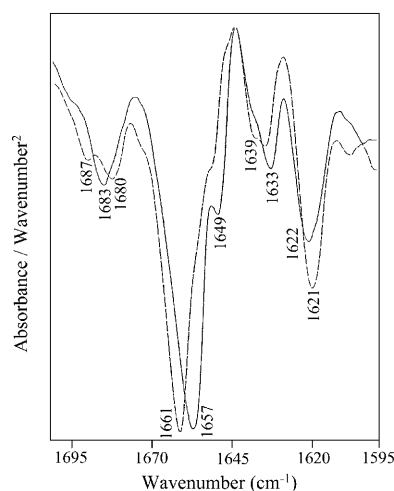


FIGURE 4 Comparison of the FTIR second-derivative spectra of alm-dUL in aqueous ( $\text{H}_2\text{O}$ ) DMPC suspension recorded above ( $30^\circ\text{C}$ , broken line) and below ( $15^\circ\text{C}$ , continuous line) the lipid phase transition temperature.

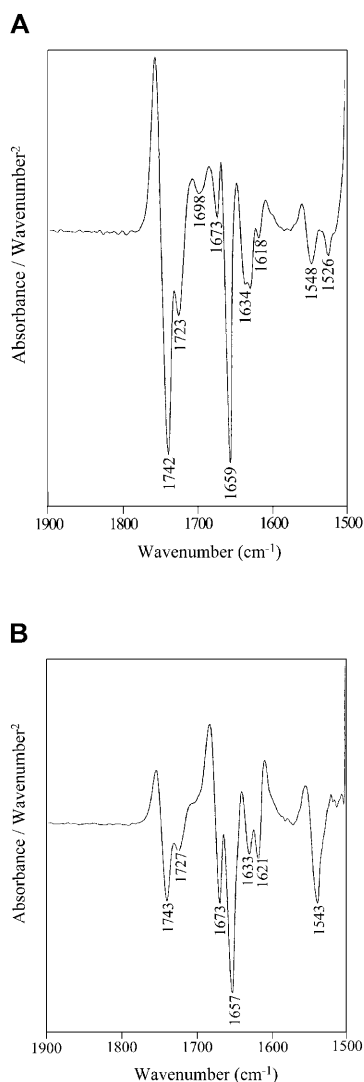


FIGURE 5 (A) Second-derivative spectrum of native alamethicin in aqueous (<sup>2</sup>H<sub>2</sub>O) DMPC suspension. Temperature = 15°C. (B) Second-derivative spectrum of alm-dUL in aqueous (<sup>2</sup>H<sub>2</sub>O) DMPC suspension. Temperature = 15°C.

dispersions were also recorded at ~1°C and 30°C, but the second-derivative spectra were similar to that recorded at 15°C (see Fig. 5). There was no significant shift of the amide I components to suggest any change in the secondary structure of native alamethicin as a function of lipid fluidity, as was previously reported for samples in H<sub>2</sub>O (Haris and Chapman, 1988).

Fig. 5 B presents the second-derivative spectrum of a sample of alm-dUL in the same conditions and recorded at 15°C. The strong amide I band is located at 1657 cm<sup>-1</sup> and minor components are also observed at 1673 cm<sup>-1</sup>, 1633 cm<sup>-1</sup>, and 1621 cm<sup>-1</sup>. The amide II band is centered at 1543 cm<sup>-1</sup>. The 1743-cm<sup>-1</sup> and 1727-cm<sup>-1</sup> components arise from lipid carbonyls. Comparison of the second-derivative spectra presented in Fig. 5, A and B, reveal significant

differences between native alamethicin and alm-dUL, especially in the frequency of the main amide I band that is shifted by -2 cm<sup>-1</sup> to 1657 cm<sup>-1</sup> for the Leu-derivative. The results obtained thus show that the main amide I component occurs at higher frequency for native alamethicin, both for samples in methanol and in aqueous lipid dispersions, compared with alm-dUL.

## DISCUSSION

Apart from transforming the partial 3<sub>10</sub>-helical character of native alamethicin into a fully  $\alpha$ -helical structure, the aim of studying alm-dUL was also to use it as a model channel-forming peptide, more akin to normal protein channels (that is without the noncoded Aib). Given the significantly increased hydrophathy index of Leu versus Aib and the greater bulkiness of its side chain, stronger hydrophobic interactions were expected both with the lipid acyl chains and between monomers. An alternative synthetic analog was also designed where all Aibs were replaced by Ala, but both its channel-forming ability and its conformational stability were rather poor (our unpublished results).

FTIR spectra of alm-dUL were obtained so that comparisons could be made with native alamethicin. Spectra obtained in methanol are similar in the amide I and amide II regions for both peptides although some small, but significant, differences were apparent. Most importantly, the amide I maximum occurs at a lower frequency for alm-dUL indicating an increase in amide hydrogen-bonding, as is also supported by a small positive shift of the amide II band toward higher frequency. As already mentioned, it is well known that an increase in amide hydrogen-bonding causes the amide I and amide II band frequencies to decrease and increase, respectively (Alvarez et al., 1987; Haris and Chapman, 1988). The fact that the amide I maximum for alm-dUL occurs at a lower frequency, and hence more closer to the absorbance expected from normal helices, suggests that it has a smaller amount of 3<sub>10</sub>-helical structure compared with the native alamethicin. It is also noteworthy that in the FTIR spectrum of alm-dUL, the amide band shape is much more symmetrical, and absorbance near 1670-cm<sup>-1</sup> and 1640-cm<sup>-1</sup> intensities are lower as compared with native alamethicin. These observations are also in favor of some conformational change and support a contribution from 3<sub>10</sub>-helical structure to the amide I band in alamethicin.

In this work, the secondary structure of both native alamethicin and alm-dUL in aqueous lipid dispersion was studied above and below the lipid phase transition. Contrasting with alamethicin, the frequency of the amide I maximum for alm-dUL was found to increase as the temperature was raised above the lipid phase transition temperature  $T_t$ . The increase in amide I band frequency suggests a weakening of hydrogen-bonding between the peptide groups above  $T_t$ . The fact that the amide I band is now located at frequency of 1661 cm<sup>-1</sup> possibly indicates an increase in 3<sub>10</sub>-helical

structure that has weaker hydrogen-bonding compared to normal helical structure. However, the changes observed could be due to other factors. For instance, the polypeptide structure might be more flexible in a fluid lipid bilayer compared with a rigid one. Increased flexibility can be related to the weakening of hydrogen-bonds, which in turn may explain the shift of the amide I band toward higher frequency. In addition, this latter effect could be due to further penetration of the peptide into the hydrophobic core and the resulting reduced hydration of the peptide groups. Reciprocally, it was recently reported that alamethicin induces different changes in lipid mobility above and below a critical peptide concentration (Kikukawa and Araiso, 2002) corresponding to a drastic change in alamethicin orientation (Huang, 2001).

Overall, the results presented here can be summarized as follows:

1. Shifts in the position of both the amide I and amide II bands clearly show that the hydrogen-bonding pattern within alm-dUL is stronger than in native alamethicin. This is found to be the case for the peptide in both methanol and phospholipid membranes. The more symmetrical amide I band for alm-dUL suggests that it has a more homogeneous hydrogen-bonding arrangement as compared to native alamethicin. The latter is likely to have a more heterogeneous structural content with peptide groups in different hydrogen-bonding pattern. Certainly, it appears that substitution of Aib residues with Leu results in a more rigid and homogeneous helical structure in alam-dUL. This conclusion agrees with previous results (Brachais et al., 1995) that showed by circular dichroism and NMR experiments that the substitution of Aib by Leu increased the  $\alpha$ -helical content and also decreased the length of alm-dUL.
2. Alm-dUL undergoes a change in its secondary structure associated with its interaction with phospholipid membranes. The main amide I band components shift to a higher frequency in DMPC membranes when above the phase transition temperature. The fact that the  $1630\text{-cm}^{-1}$  and  $1657\text{-cm}^{-1}$  components shift in membrane systems indicate that both of these structures interact with lipid membranes. It is probable that the alm-dUL peptide insertion into the lipid membrane is facilitated when the latter is in a fluid state. This insertion, which is driven by voltage changes below a critical peptide concentration, is a prominent step in channel buildup (Helluin et al., 1997).

We conclude that substitution of Aib residues with Leu produces a more stable structure for the monomers that display strong interaction with phospholipid membranes. A NMR study in SDS micelles (Brachais et al., 1998) concluded also that Alm-dUL helices showed regular structure along their axes that are lightly bent near the Pro<sup>14</sup> although a higher flexibility was observed in MeOH. These structural

findings are correlated with functional data comparing channel-forming activity of both peptides in planar lipid bilayers (Fig. 2). It thus appears that some structural flexibility in addition to an adequate peptide length/bilayer thickness match favors long-lived channels. In a previous article, Brachais et al. (1995) considered that alamethicin was largely helical with several  $3_{10}$  parts in the C-terminal, whereas Alm-dUL seemed to be exclusively in  $\alpha$ -helical conformation according to NMR experiments. Thus the authors claimed that Alm-dUL would be certainly shorter than alamethicin and consequently less able to cross completely the membrane under applied voltage. This parameter would be responsible of faster fluctuations of ion channels induced by Alm-dUL in planar lipid bilayers. Further molecular modeling study (Brachais et al., 1998) confirmed the shortening of Alm-dUL compared to the one of Alm (26.5 Å against 29 Å).

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