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REVIEW

Recent Results of Alamethicin Research

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Contents

1. Introduction. – In 2007, *Leitgeb et al.* [1] presented a historical overview about research on alamethicin (ALM), discussing the discovery, primary structure, biosynthetic pathway, structural and conformational properties, channel formation and biological activities of ALM and its synthetic analogues.

ALM is a linear peptide belonging to the family of peptaibols. ALM consists of 20 amino acid residues with phenylalaninol (Pheol) at the C-terminus and an acetylated N-terminus. The originally described compound is composed of two major groups named ALM F30 and ALM F50 and a minor component (ALM F20) based on their retention factor values in tissue liquid chromatography on acidic silica gel [2].

The organism producing ALM – originally named as compound U-22324 – was initially reported to be '*Trichoderma viride*' strain NRRL 3199 [3]. However, the producer was recently reidentified as *T. arundinaceum* from the so-called "Brevicompactum clade" of the genus *Trichoderma* [4]. Furthermore, all other *Trichoderma* species known to produce ALM (*T. brevicompactum, T. protrudens, T. turrialbense*) also belong to this clade [5] [6].

Biosynthesis of ALM is independent from the general, ribosomal peptide-translation pathway. Similarly to all other peptaibols, ALM is synthesized in a microheterogenous mixture on a multi-enzyme complex from the family of nonribosomal peptide synthetases (NRPSs), that are large multifunctional enzymes with modular structure, assembling the peptides using a protein template via the multiple-carrier thiotemplate mechanism [7]. Most of the NRPSs terminate peptide synthesis with the thioesterase domain, which is however, missing from the peptaibol synthetase of *Trichoderma virens* (TPS). *Manavalan et al.* [8] presented a three-dimensional (3D) model of the reductase domain of TPS, which is playing a role in

the release of the mature peptide products reductively with the aid of a NADPH cofactor. The authors identified the binding sites of the probable linear substrates including ALM, antiamoebin I, chrysopermin C, gramicidin and trichotoxin within the modeled reductase domain using multiple docking approaches.

As summarized by *Leitgeb et al.* [1], ALM has a predominantly α-helical conformation with a small distortion in the structure generated by the Pro residue at the 14^{th} position, which is referred to as proline-kinked α-helical conformation. ALM is capable of binding to the surface of lipid bilayers and insertion into the membranes, which is depending on a series of parameters like elasticity, structure and hydration level of the lipid bilayer, peptide concentration, peptide/lipid molar ratio as well as temperature [1]. The channel structures formed by ALM and its analogues were examined and the residues important for ALM-bilayer interactions, channel formation and channel stabilization could be identified. Molecular modeling studies of ALM bundles embedded in lipid bilayers revealed important data about the structural and dynamic features of ALM [1].

The wide spectrum of biological activities exhibited by ALM includes antimicrobial effects, elicitation of systemic plant-defense responses, tissue damage in insect larvae as well as cytolytic activity towards mammalian cells [1].

The aim of this review is to summarize the significant amount of information about ALM which accumulated in the literature during the past 5 years since the publication of the review of *Leitgeb et al.* [1].

2. Recent Results about Alamethicin-Membrane Interactions – ALM is perhaps the most studied pore-forming antimicrobial peptide (AMP), which is primarily due to its use as a model of voltage-gated ion-channel conduction. It can

bind to the surface of different lipid bilayers, and after that it can insert into them with multiple open states regarding to its oligomerization level. The dependence of this process from several parameters such as temperature, peptide concentration, peptide/lipid (P/L) molar ratio and the type of the lipid bilayer was described and reviewed in details by *Duclohier* and *Wróblewski* [9]. The present review will focus on recent results about ALM-membrane interactions summarizing the novel experimental setups in bilayer techniques, pore modulation and regulation possibilities, features of the lipid bilayer environment of pores as well as biosensing opportunities. ALM is applied these days as an "etalon" or control in several cases for the characterization of conduction features of novel pore-forming molecules [10−12]. Its fusogenic effect was also tested on lipid vesicles as one of the gold standards of membrane activity, but it was completely inactive in the applied experimental setup [13].

2.1. Experimental Designs to Test Pore Formation of Alamethicin. In the case of traditional black lipid membranes (BLMs), the lipids are associated solely via relatively weak intermolecular interactions. Therefore their lifetimes are significantly limited and long-term monitoring of ALM ion channel activity as well as effective involvement in biosensing applications is not possible. There are some strategies to prolong the lifetime of the membrane test systems including polymerization, hydrogel support and miniaturized apertures. *Heitz et al.* [14] described mixed BLMs prepared using bis-dienoyl phosphatidylcholine and diphytanoyl phosphatidylcholine, which exhibit greatly enhanced stability upon photopolymerization (4 days) relative to unpolymerized BLMs (4 hours). In this experimental setup, ALM − for which a relatively high membrane fluidity is required − could maintain its pore forming activity and showed a characteristic series of its subconductance states, but higher

currents were measured on it due to increases in size, number and/or frequency of pores [14]. The other strategy for the extension of membrane lifetime is the maintenance of the solvent annulus at the aperture boundary. For this purpose, *Jeon et al.* [15] explored ways by encapsulating and conjugating the BLM within hydrogels and formed it on a glass substrate. Using this approach, continuous electrical and optical monitoring of membranes was achieved for 12 days. ALM was able to successfully incorporate into these types of membranes and the measured channel conductances were similar to those measured prior to polymerization as well as in conventionally formed membranes. *Harriss et al*. [16] developed a new method for the generation of artificial lipid bilayers, which involves contacting the monolayers formed on a nanoliter aqueous droplet and a hydrogel layer immersed in an oil/lipid solution to create a bilayer. This method enables both single-molecule fluorescence Ca^{2+} flux imaging and single-channel electrical recording from the bilayer. This simultaneous measurement allowed observation of multiple conductance steps originating from a single ALM pore. The individual pores switch rapidly between distinct conductance states which correlated with fluorescent measurements, confirming that multiple conductance states could be originated even from a single pore. *Maurer at al.* [17] brought together several previously developed features into a single microfabricated structure, creating large-sized orifices on silicon nitride substrates in combination with an agarose backing layer. The created membrane system showed enhanced stability (4 days) and was appropriate for the measurement of single channel conductance and sub-conductance states of ALM at levels similar to that observed in a vertical painted BLM. The presence of automated laboratory instruments in the research work can also help in the formation of lipid bilayer systems through a „hand-free" way [18]. Using an automated liquid-handling

equipment, a lipid membrane platform was developed, which can allow the high throughput measurement of a large number of different samples simultaneously by adopting a vertical orientation of the monolayer contacting method. The platform was succesfully tested for ALM membrane pores and it was suggested as an excellent tool for the high throughput ion channel drug screening.

Krauson et al. [19] designed a novel set of analytical tools, which allow to examine the pore properties of ALM using fluorescent head group-labeled lysolipid monomers. This test system represents other approach than the conductance recordings and is able to measure four properties of peptides at very low peptide to lipid ratio. These properties are the following: i) the potency of peptide pores, ii) the continued presence or absence of peptide pores at equilibrium, iii) intermembrane exchangeability of pore-forming peptides, and iv) disrupting effect at equlibrum. Theoretically, 4–20 peptides (1–2 pores) were the lowest detection limit of the described technique.

2.2. Pore Modulation Effects. Artificial lipid membranes (mainly the BLMs) have been widely used to reconstitute and study ALM channels, as they allow electrochemical access to both sides of the ion channel-membrane complex, where multiple single-channel conductance levels can be recorded, which reflect uptake and release of individual monomers within trans-membrane conducting bundles [20].

The pH can also play an important role in modulation of electric features of zwitterionic-based artificial lipid membranes. This pH effect on transmembrane electrical properties of membranes was examined in the work of *Chiriac* and *Luchian* [21] by evaluating the transport properties of embedded ALM oligomers over a wide range of pH values. Their investigation involved single ALM oligomers, and they demonstrated an unexpected, non-monotonic dependence of the single channel

electrical conductance in the function of pH. At extreme acidic values, the electrical conductance of the first and the second sub-conductive state of the ALM oligomer is reduced with about 20% and 11%, respectively, as compared to moderately acidic pH values. The proposed mechanism is that the partitioning of hydrogen and hydroxide ions between both membrane sides leads to changes of the overall superficial charge on the membrane and of the membrane dipole potentials, allowing modulation of the local concentration of cations near the ALM oligomer, thereby facilitating their hopping across the channel. Additionally, pH modulation has an effect among lipid head groups of membrane monomers, which could alter the curvature stress in the bilayer, and this would lead to a visible mechanical constriction of the ALM pore manifested by a drop in its conductance at moderated pH values [22] [23]. Amphiphilic compounds can provide reliable tools to understand the effects of asymmetry in the electrostatic features of a reconstituted lipid membrane on the dynamic properties of ALM. They preferentially partition into a single membrane leaflet, added vectorially to the membrane opposite to the side of ALM insertion to controllably alter the dipole and surface potential and provide a quantitative evaluation of the effects of asymmetric bilayer electric potentials on peptide function. The styrylpyridinium dye RH 421 absorbs on the lipid bilayer interfacially and increases the membrane dipole potential, which could decrease the membrane activity of ALM most likely via an elevated repelling influence upon the positively charged N-terminus, as it moves across the interfacial region and penetrates the lipid membranes [24]. Other amphiphiles like phlorizin and sodium dodecyl sulfate partition also preferentially into a single membrane leaflet, when added vectorially to the membrane opposite to the side of ALM insertion to controllably alter the dipole and surface potential. It was described that these

potentials generated asymmetry between the both sides of a membrane and give a trans-bilayer driving force, which strongly modulates the ALM activity and ion transport properties. Specifically, phlorizin (a dipole potential lowering agent) augmented the ALM activity, while in the case of RH-421 the opposite was observed because of the membrane dipole potential enhancing effect of the amphiphile. The ion conductance of oligomeric ALM in its various substates is increased and decreased by the injection of phlorizin and RH-421, respectively on the trans sub-phase in the same manner. Additionally, after termination of electric modulatory effects through the membrane bilayer applying the anionic detergent sodium dodecyl sulfate on both sides of the membrane, its mechanical effect on peptide activity remained still active [25].

Aliverdieva et al. [26] studied the initial steps of pore formation in rat liver mitochondria preparations as a potassium transmembrane current sensor generating transmembrane potential to evaluate the molecularity of the rate-limiting step of pore formation by ALM *in situ*. The applied transmembrane current depends linearly on the degree of steady-state activation of the model membrane respiration. This activation was limited by association of monomers of ALM to pre-pore and the order values coincided with molecularity or the degree of pre-pore oligomerization, and it was close to 2 formed by ‘‘dimers’’. *Vedovato* and *Rispoli* [27] applied a novel technique to study ALM under strict physiological conditions, which consisted of inserting the peptides into cell plasma membranes where all the endogenous current sources were blocked. During the conductance measurements the general single-channel events were recorded at low ALM concentrations (<250 nM) according to the different number of helices constituting a single channel. However, after four-fold reduction of peptaibol concentration, the current fell from several hundreds of

pA to the single channel level and after the removal of ALM it fell to zero within a few hundred ms, which shows the cooperativity and fast speed of disaggregation.

2.3. Size of Alamethicin Pores and the Effect of Lipid Environment on Pore Formation. Katsu et al. [28] applied calcein as fluorescence permeability marker simultaneously with K^+ release measurement to study the ALM permeability in liposomes. This technique can provide significant information about the sizes of pores based on the differences in the rates of release of K^+ and calcein, although their results were confirmed also by conducting an osmotic protection experiment. They found that amphiphilic peptides, including ALM, formed relatively large pores (belonging to the type 3 cluster) in liposomal membranes (>0.67 nm radius) and did not form small above-mentioned voltage-gated ion-channels in the applied liposomal membrane. ALM-induced holes were directly visualized in gel dipalmitoylphosphatidylcholine membranes by atomic force microscopy and both the structure of pores and the membrane around the transmembrane peptides were characterized [29]. According to the calculation of pore perimeters it was observed that at the lower ALM concentration (1 mol%) the number of aggregated peptaibol molecules varied from 30 to 300 molecules per hole, while at the higher contcentration of 4 mol% it reached a value of up to 1200. These detected pores were referred as ‘defects’ because of their relatively large diameter, although they may be closely related to, or indistinguishable from pores. Furthermore, lipid membrane phase behaviour was altered in the vicinity of these defects determined by differential scanning calorimetry [29]. This phenomenon was induced by ALM molecules, which influence the heat capacity profiles of membranes perhaps through the distribution of lipids with different state or chemical nature affecting the elastic constants in the pore environment to bring the membrane system close to a melting

transition. It was concluded that in native biological membranes this process could play an important role in controlling the physical features of lipid–protein complexes [30]. This membrane alteration effect was also observed using dilatometry and small-angle X-ray diffraction of dioleoyl phosphatidylcholine bilayers, which manifested in the form of membrane thinning, increase to the lateral area per lipid and a decrease in the bending rigidity of the membranes. Moreover, it was suggested that softening of the lipid bilayer significantly influences lipid-mediated peptide-peptide interactions in the pore formation [31]. The importance of the lipid-peptide interaction and of the chemical nature was also demonstrated during the examination of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine liposomes by cryo-transmission electron microscopy in combination with turbidity and leakage measurements, where it was proved that cholesterol modulates the membrane perturbing effect of ALM [32]. The ALM concentrations induced membrane rupture and fusion of cholesterol-supplemented liposomes, but did not cause any discernible structural alterations in systems containing cholesterol-free liposomes. *Bertelsen et al.* [33] also reinforced the importance of the full system in structural studies of membrane peptides due to the effects of the peptid-lipid interactions. They concluded that especially the fold and possibly the oligomerization of ALM may be altered if ether lipids are used instead of the naturally occurring ester lipids in artificial lipid system.

2.4. Practical applications. Based on the accumulated knowledge about the pore forming properties and the regulation possibilities of the assembled channels it is possible to integrate this biological construction into biotechnological and biosensing appications. *Mayer et al.* [34] described the use of a synthetically modified ALM in planar lipid bilayers to quantify protein-ligand interactions. The technique is based on the measurement of ion flux, which appears as reduced

conductivity through the pores where the monomers contain covalently attached benzenesulfonamide group on the C-terminus. The linked group is a substrate of the carbonic anhydrase II enzyme and after the protein-ligand interaction it triggers the reduction of the ion current between the two sides of the membrane at a measurable level. If the recorded currents were time-averaged, the amount of transported charge before and after the addition of the enzyme was distinguishable to serve a promising amplifying method for sensing enzymatic reactions like the widely applied enzyme-linked immunosorbent assay (ELISA). Bionanoelectronic interfaces involve functional integration of nanomaterials with membrane proteins and peptides, and ALM-oligomerized pores are potential candidates for this purpose. A novel construction was published using silicone nanowire transistor, which contains nanowire covered by lipid membrane-incorporated ALM molecules [35]. This membrane-shielded wire setup was able to enable and control ionic to electronic signal transduction by using voltage-gated and chemically-gated ion transport through the membrane pores. The constructors of the device exploited that at negative membrane potentials ALM does not span the entire transmembrane thickness, while at positive membrane potentials it penetrates the membrane completely and forms a channel, which allows the opening and closing of the pores in the lipid bilayer like turning on and off a simple electrical switch. Furthermore, the closing state of the pores was completely identical with the ALM-free bilayer-coated nanowire design. Besides the electronic control, the ALM pores are able to actuate also with mechanical effect according to the interaction between the lateral pressure profile and the ALM pore formation [36]. A laser-drilled, single-crystal quartz substrate was used efficiently as a mechanotransductive interface to influence the diameter of the ALM pores by exciting opposing thickness-shear modes [37]. The

increased mechanical effects on the lipid membrane increased the larger conductance states of the incorporated channels: the mechanical tension changes were able to transduce into an electrical signal response, thereby serving a new way for the utilization of the piezoelectric-based membrane system as a mechano-sensitive biosensors.

Kropacheva and *Raap* [38] reported the biotechnological application of ALM-membrane system for improving the bilayer-separated enzyme reactions using a new strategy for increasing the substrate permeability through membrane bilayers. They studied the enzymatic hydrolysis of Na-benzoyl-l-arginine-p-nitroanilide hydrochloride by liposome-encapsulated trypsin. The substrate which was applied at the outer side of the membrane showed low permeability on the liposome. In this model system, approximately seven-fold increase of the enzymatic reaction rate was observed in the presence of the peptaibol, when compared with the control where the peptaibol molecules were not added to the liposomes. These results suggest that ALM could be used in reactions catalyzed by vesicle- or cell-entrapped enzymes as a substrate carrying agent.

3. Recent Results about Structural and Conformational Properties of Alamethicins and their Channels. – The review of *Leitgeb et al.* [1] discussed previous results about the 3D structure and the different conformational features of ALM and its analogues, derived from a variety of experimental techniques, including X-ray diffraction (XRD), Nuclear Magnetic Resonance (NMR), Circular Dichroism (CD), Raman and Fourier Transform Infrared (FTIR) spectroscopies, as well as obtained by various theoretical methods, comprising distance geometry (DG), simulated annealing (SA) and molecular dynamics (MD). The earlier experimental

and theoretical studies were performed in different environments under various circumstances, i.e. in the crystalline phase, in aqueous solution, in several organic solvents, in the presence of various micelles as well as in different membranes. As summarized by *Leitgeb et al.* [1], the structural investigations of ALM channels and ALM-analogue channels were predominantly carried out using different MD simulations. The present review is discussing the recent developments in this field.

3.1. X-Ray Diffraction Studies. Crisma et al. [39] examined the 3D structure of a spin-labeled ALM analogue ([TOAC^{16},Glu(OMe)^{7,18,19}] ALM F50/5, Table 1) by means of XRD, and two individual molecules were identified. Both of them could be characterized by a bent, predominantly α-helical conformation, however, they showed differences with regard to their intramolecular H-bonding patterns, as well as to their bend angles detected near the Pro^{14} amino acid. For the helical structures of two molecules, the side-chains of three γ-methyl glutamate (Glu(OMe)) residues were found on the same side, whereas the most hydrophobic side-chains and the 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) residues were located on the opposite side, resulting in a hydrophilic convex as well as a hydrophobic concave face of both peptides. Based on the results it was concluded that the overall conformational features of two crystallographically independent molecules were found to be similar to those observed previously for the three individual ALM molecules identified in the asymmetric unit cell of crystals [40].

In the XRD study performed by *Qian et al.* [41], the electron density profile of transmembrane pore induced by the ALM molecules was reconstructed, applying fully hydrated multiple bilayers of the ALM-lipid mixtures. The results derived from this XRD measurement pointed out unequivocally that the structure of ALM-induced pore corresponded with the “barrel-stave” model composed of eight ALM helices. In

the course of this study, the ALM pore was directly imaged for the first time by reconstructing the electron density of pore on the basis of XRD measurement.

3.2. Spectroscopic Studies. Salnikov et al. [42] investigated the structural features of ALM F50/7 by ^{15}N and ^{31}P solid-state NMR spectroscopy, with regard to its membrane-bound state, applying two types of lipid bilayers, i.e. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) membranes. As the NMR data indicated, the structure of ALM inserted into the lipid bilayers was characterized by a mixed α-/3_{10}-helical conformation. Nevertheless, this NMR study led to the observation that ALM adopted a more tilted helical structure for the DMPC membrane as compared to that determined for the POPC membrane.

The membrane-bound conformation of ALM F30/3 was examined by *Bertelsen et al.* [43], using ^{2}H Magic-Angle Spinning (MAS) solid-state NMR spectroscopy, and it was assumed that ALM adopted an α-helical structure. In this study it was demonstrated that ^{2}H MAS NMR experiments could be applied as a novel method to obtain fast and accurate conformational constraints concerning the peptaibol molecules in their membrane-bound environments. In a subsequent study, *Bertelsen et al.* [44] applied ^{1}H-^{15}N and ^{2}H solid-state NMR spectroscopy in order to derive residue-specific information about the conformational dynamics of ALM F30. Based on the NMR data, detailed information was provided with regard to the tilt angle of helix, to wobbling, as well as to the oscillatory rotation around the helix axis for the membrane-bound conformation of ALM. Comparing the results obtained by this NMR study with those derived from the coarse-grained (CG) MD simulation performed on ALM molecules [45] it was concluded that the experimental and theoretical data concerning the dynamic model were in good agreement with each

other. In a more recent study, the interplays between ALM F30 and ether/ester phosphocholine lipid bilayers were investigated by *Bertelsen et al.* [33] using CD spectroscopy to determine the secondary structure of ALM, as well as applying oriented solid-state NMR spectroscopy to characterize the conformational and dynamic features of the peptide in detail. As the results indicated for ALM, differences were observed with regard to the secondary structure as well as to the temperature-dependent membrane anchoring, taking into account the two types of lipid bilayers. The CD spectra revealed that ALM did not adopt a predominantly α-helical conformation either in ether bicelles or in ester bicelles, and based on this observation it was suggested that the lipid environments affected the structural and conformational properties of membrane-bound ALM.

Kouzayha et al. [46] studied the interactions of ALM with Langmuir monolayers and multilamellar vesicles by IR and NMR spectroscopies in order to identify the role of peptide conformation played in the interplays evolved between ALM and lipids. At the air-water interface it was observed that ALM could be characterized by an α-helical structure, and the α-helix axis of the ALM peptide was preferentially oriented parallel to the air-water interface. Studying the interactions of ALM with DMPC and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) monolayers led to the observations that for the DMPC monolayers the ALM-ALM interactions were more stable than the interplays between the peptide and lipid, whereas for the DPPC monolayers the DPPC-ALM interactions were found to be more stable as compared to the interplays observed between the ALM peptides. The results obtained by IR and NMR measurements indicated that the ALM interacted with the DMPC multilamellar vesicles via hydrophobic interactions with alkyl chains.

Ye et al. [47] applied Sum Frequency Generation (SFG) vibrational spectroscopy and Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR), to characterize the interplays between ALM and various lipid bilayers without the presence of a membrane potential. The results revealed that different peptide-lipid interactions could be observed for the fluid-phase lipid bilayers, in comparison with those found for the gel-phase ones. In fluid-phase lipid bilayers, ALM was characterized by a mixed α-/3_{10}-helical conformation, and its N-terminal helical part was found to be more tilted than its C-terminal helical part, with respect to the surface normal. In contrast, ALM molecules could not insert significantly into the gel-phase lipid bilayers, however, they lay down and/or aggregated on the surface of this type of lipid bilayers. In a subsequent study, *Ye et al.* [48] investigated the interactions between ALM and POPC lipid bilayers in the presence of a membrane potential, using SFG vibrational and ATR-FTIR spectroscopies. For ALM molecules, the orientation in POPC lipid bilayers was determined at different pH values, where the tilt angles were calculated in the case of N-terminal α-helical part, as well as of C-terminal 3_{10}-helical part, respectively. Based on the results it was observed that the values of tilt angles were decreased as a function of the increasing pH values. Overall it was concluded that the change in the membrane potential modulated by the alteration of pH values produced an effect not only on the orientation of peptide, but also on the bend measured between two helical segments.

Stella et al. [49] synthesized a set of ALM F50/5 analogues and they examined the position and orientation of these peptides in the membrane environment by ATR-FTIR measurements. For two of these analogs ([Glu(OMe)^{18,19}] ALM F50/5 and [Glu(OMe)^{7,18,19}] ALM F50/5, Table 1), two and three Gln amino acids were substituted by Glu(OMe) residues, respectively. For the other two ALM analogues

(Fmc-[Glu(OMe)^{7,18,19}] ALM F50/5 and [Glu(OMe)^{7,18,19}] ALM F50/5-Fmc, Table 1), relative to the [Glu(OMe)^{7,18,19}] ALM F50/5, a 9*H*-fluoren-9-ylacetyl (Fmc) group was introduced either at the N-terminal end or at the C-terminal end of the peptide. This ATR-FTIR study demonstrated that the N-terminal part of peptides was inserted into the membrane, while their C-terminal part was found to be exposed to the outer aqueous phase, and furthermore, different orientations of molecules were observed with respect to the normal of membrane. For the ALM F50/5 and its analogues the CD spectra proved to be almost identical, indicating that the sequence modifications of parent peptide did not affect the helical structure and the conformational features of ALM F50/5.

Peggion et al. [50] performed a detailed conformational analysis in solution by means of CD, FTIR and NMR spectroscopies for the [Glu(OMe)^{7,18,19}] ALM F50/5 analogue (Table 1) and for its four derivatives containing TOAC residues instead of Aib amino acids ([TOAC^{1},Glu(OMe)^{7,18,19}] ALM F50/5, [TOAC^{8},Glu(OMe)^{7,18,19}] ALM F50/5, [TOAC^{16},Glu(OMe)^{7,18,19}] ALM F50/5 and [TOAC^{1,16},Glu(OMe)^{7,18,19}] ALM F50/5, Table 1). The CD spectra of five ALM analogs were found to be very similar to that observed for ALM, indicating that these peptides adopted a highly helical conformation, containing a large amount of α-helix, as well as a small amount of 3_{10}-helix. Based on the data derived from the FTIR measurements it was concluded that the preferred conformation of peptides could be characterized by a highly folded structure, which was stabilized by intramolecular H-bonds. The NMR study carried out on the [Glu(OMe)^{7,18,19}] ALM F50/5 analogue revealed also that this peptide was largely helical. Overall, the afore-mentioned spectroscopic studies led to the conclusion that neither the Gln vs. Glu(OMe) nor the Aib vs. TOAC

replacements produced any significant effects on the preferred conformation of the parent peptide, ALM.

The self-aggregation of a spin-labeled ALM F50/5 analogue ([TOAC^{16},Glu(OMe)^{7,18,19}] ALM F50/5, Table 1) in egg phosphocholine (ePC) vesicles was studied by *Milov et al.* [51], applying the Pulsed Electron-Electron Double-Resonance (PELDOR) technique. The data derived from this experiment pointed out that the ALM analogue self-aggregated in ePC vesicles, and this aggregates consisted of about four peptides. In this study it was demonstrated that the PELDOR method could be applied to determine long-range distances between transmembrane helical peptides. Subsequently, *Milov et al.* [52] used the PELDOR technique, to characterize the conformation of a further spin-labeled ALM F50/5 analogue ([TOAC^{1,16},Glu(OMe)^{7,18,19}] ALM F50/5, Table 1) for the non-aggregated peptides in pure ePC and in solvents with various polarity, as well as for the aggregates of peptides in hydrated ePC vesicles. The results pointed out that the intramolecular distances between the two TOAC residues were in excellent agreement with that calculated based on the α-helical conformation of this ALM analogue. Furthermore it was concluded that the above-mentioned distances are independent of the applied experimental conditions, namely, they depended neither on the aggregated state of the peptide nor on the polarity of the solvent. Overall, the data obtained by the PELDOR spectroscopy indicated that this spin-labeled ALM analogue could be characterized by a predominantly α-helical conformation. Additionally, for the aggregated peptides it was observed that the hydrophilic residues were located on the same side of the α-helical structure, resulting in an amphipathic character of aggregated molecules. *Jose et al.* [53] determined nitroxide to peptide NH proton distances derived from Paramagnetic Relaxation Enhancement

(PRE) measurements for [TOAC^{1},Glu(OMe)^{7,18,19}] ALM (Table 1), which were compared with the intramolecular distances obtained by the previously-mentioned XRD study performed on [TOAC^{16},Glu(OMe)^{7,18,19}] ALM F50/5 [39]. The results led to the conclusion that the distances identified via PRE measurements were in close agreement with those obtained from the XRD-based model, indicating that the conformational properties of these ALM analogues were similar to each other in solution and in the crystalline phase. Additionally, it was suggested that these structural information regarding the intramolecular distances could be applied as long-range distance restraints in the case of NMR-based structure determination.

Maisch et al. [54] synthesized a series of ALM analogues (Table 1), in which the Aib amino acids located in positions 5, 10 and 16 were substituted either by the (*R*)- or by the (*S*)-stereoisomer of trifluoromethylalanine (CF_3-Ala) residues, respectively, resulting in six different analogues of the parent peptide. The structural features of these ALM analogues – regarding their conformation, alignment and dynamics – were examined by solid-state ^{19}F NMR spectroscopy in DMPC bilayers. For ALM and its six analogues, the CD measurements performed in solution revealed similar spectra, indicating that these peptides could be characterized by a high helical content, as well as pointing out that the replacement of Aib amino acids by the CF_3-Ala residues did not perturb the backbone conformation of ALM analogues. On the basis of the results it was concluded that the N-terminal part of ALM adopted an α-helical conformation, which showed a transmembrane alignment with a characteristic tilt angle in DMPC membranes.

The structural properties of another set of ALM analogues were investigated by *Noshiro et al.* [55] using CD spectroscopy in the presence of POPC vesicles. For the parent ALM analogue (HG-ALM, Table 1) a His residue was attached via a Gly

spacer at the N-terminal end of the ALM sequence, while for its three derivatives HG-[Leu]ALM, HG-[Nva]ALM and HG-[Nle]ALM (Table 1) all the Aib amino acids were substituted by Leu, norvaline (Nva) and norleucine (Nle) residues, respectively. In the case of all four peptides, the CD spectra indicated a helical structure in the absence of Zn^{2+}, however, it was observed that the addition of Zn^{2+} caused changes for HG-ALM and HG-[Nva]ALM, while did not cause significant changes for HG-[Leu]ALM and HG-[Nle]ALM, regarding their CD spectra. Based on these results the helical contents of the latter two peptides were found to be similar to those found for the former ones, but in the case of HG-ALM and HG-[Nva]ALM, a significant enhancement of helical conformations was detected in the presence of Zn^{2+}.

Hjorringgaard et al. [56] synthesized a new class of templated ALMs, for which the peptides were conjugated to the α- and β-cyclodextrins either from the N-terminal end or from the C-terminal end. On the basis of CD spectra it was observed that the secondary structure adopted in solution was similar for these ALM:CD conjugates to that found in lipid, nevertheless, the membrane-induced structure of templated ALMs proved to be the same as that of ALM. In order to determine the orientation of peptides in lipid, Oriented Circular Dichroism (OCD) measurements were performed. The results indicated a more tilted state of α-helices for the N-terminally linked ALM conjugates (ALMNαCD and ALMNβCD), while perpendicular helices were observed for the C-terminally linked ALM conjugates (ALMCαCD and ALMCβCD) as well as for ALM.

3.3. Combined Spectroscopic and Theoretical Studies. Dittmer et al. [57] studied the incorporation and the membrane-bound conformation of ALM in lipid bilayers by means of ^{1}H liquid-state NMR spectroscopy combined with MD

simulations, applying 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine/1,2-dihexanoyl-*sn*-glycero-3-phosphatidylcho-line (DMPC/DHPC) bicelles. Based on the distance restraints derived from the Nuclear Overhauser Effect (NOE) and PRE it was observed that the structure of ALM in the bilayers could not be characterized by a single conformational model because of the dynamics and heterogeneity of investigated system. These NOE and PRE data were then compared with the results obtained by MD simulations performed on an ensemble of ALM peptides in DMPC bilayer. Overall it was concluded that the combination of NMR spectroscopy and MD simulations provided more information about the flexibility of ALM in the membrane environment as well as of ALM-lipid interactions.

For three spin-labeled ALM analogues ([TOAC^{1},Glu(OMe)^{7,18,19}] ALM F50/5, [TOAC^{8},Glu(OMe)^{7,18,19}] ALM F50/5 and [TOAC^{16},Glu(OMe)^{7,18,19}] ALM F50/5, Table 1), Electron-Spin-Resonance (ESR) and PELDOR measurements were carried out by *Milov et al.* [58] in various solvents in order to study the self-assembly of helical peptides. According to the different polarity of solvents, the ALM analogues showed distinct characteristics concerning their self-assembly behavior, namely, the peptides formed homogeneous solutions in polar solvents, while they formed aggregates in weakly polar solvents. On the basis of data it was estimated that the average number of ALM molecules found in the aggregates was less than nine. Applying the results derived from the experiments, a MD-based model was proposed for the aggregates of ALM analogues, which composed of two ALM tetramers parallel to each other, and both of them consisted of four helical peptides associated in a ‘head-to-tail’ fashion. Additionally it was concluded that the ‘head-to-tail’ arrangement observed in the aggregates was in agreement with the packing of peptides detected in the crystal structure of [TOAC^{16},Glu(OMe)^{7,18,19}] ALM F50/5

[39]. In a subsequent study, for three spin-labeled ALM analogues mentioned above, the supramolecular structures of self-assembled peptides were examined by *Milov et al.* [59] in their membrane-bound states using PELDOR and Electron Spin Echo Envelope Modulation (ESEEM) spectroscopies, as well as molecular modeling methods. Based on the data derived from PELDOR measurements, distance distribution functions were obtained with regard to the intermolecular distances calculated between the TOAC residues of aggregated peptides. Nevertheless, the number of ALM analogues forming the aggregates was determined, and it was estimated that the aggregates composed of ca. 4 peptides. Applying the ESEEM experiments, the penetration depths and the water accessibility were estimated, concerning the various spin-labeled positions. Additionally, a molecular modeling study was performed using distance restraints derived from the intermolecular distances between the TOAC residues, in order to investigate the interactions between the helices. This model of supramolecular ALM tetramer consisted of four molecules packing together in a parallel fashion, which were characterized with a slightly tilted α-helical conformation. On the basis of geometrical data, a penknife model was suggested, in which two peptides were aligned parallel to each other, while the arrangement of two other peptides could be characterized by an angle.

Thogersen et al. [45] carried out MD simulations combining CG and all-atom (AA) MD calculations to comprehensively study the processes with regard to the peptide aggregation as well as to the pore formation for the ALM molecules in hydrated DMPC lipid bilayer. Based on the CG simulations it was observed that the peptides aggregated spontaneously in the lipid bilayer, and it was found that these aggregates did not compose of a specific number of molecules. Nevertheless, the individual ALM peptides adopted distinct structures with respect to the lipid bilayer,

such as surface-bound and membrane-spanning states, and the helix axis was orthogonal for the former conformation while it was parallel for the latter one, relative to the normal of membrane. In addition to the aggregation mentioned above, during the CG calculations, transitions were also detected between the surface-bound and the membrane-spanning states of molecules. On the basis of subsequent AA simulation it was observed that water pores were formed across the lipid bilayer, and the membrane leaked water molecules around the ALM aggregates. Furthermore, this AA calculation led to the observations that not only the form of clusters differed from the model suggested previously, in which the α-helices formed a symmetric channel, but significant deviations from the perfect α-helical structure were detected also for the structure of individual ALM molecules. Beside the afore-mentioned MD simulations, solid-state NMR measurement was carried out, and the results supported the conclusion obtained by the MD calculations, i.e. that the ALM peptides adopted a diverse set of conformations in the lipid bilayer.

3.4. Theoretical Studies. Zhan and *Lazaridis* [60] performed MD simulations on ALM applying an implicit membrane model with membrane dipole potential in order to investigate the interactions between ALM and a zwitterionic bilayer. In the course of this theoretical study, the effects of membrane dipole potential produced on the orientation and the binding energy of peptides were determined. Starting either from parallel or from perpendicular orientation of ALM, with respect to the membrane plane, the peptides adopted certain tilt angles during the MD calculations. Additionally, a decreasing tendency was observed for the binding affinity of ALM as a function of the increasing dipole potential. Based on the results it could be concluded that not only the peptide orientation in the membrane, but also the binding energy of peptides could be affected by the dipole potential.

MD simulations were carried out by *Mihajlovic* and *Lazaridis* [61] on ALM monomers and oligomers inserted into two types of preformed pores (toroidal and cylindrical) to examine their preference for the different pore shapes. This study led to the observations that at the end of simulations an ALM hexamer preserved the starting cylindrical pore if the Gln^7 residues were located within the pore, however, the pore was found to be closed if the afore-mentioned Gln amino acids were not oriented towards the pore interior. These findings were in agreement with the prevailing view that ALM forms channel according to the most widely accepted "barrel-stave" model. On the basis of MD simulations it was deduced that the secondary structure of peptides was more stable for the ALM hexamer embedded into a cylindrical pore, as compared to that observed for the ALM tetramer embedded into a toroidal pore. In a subsequent study, MD simulations were performed by *Mihajlovic* and *Lazaridis* [62] on an ALM analogue in which the Gln^7 was replaced by Lys (ALM Q7K, Table 1), in order to study the formation of pores by this peptide, as well as to examine how the charged residues affected the pore formation. The same simulation strategy was applied in the course of these MD calculations as previously for ALM [61], and the peptides were inserted into a preformed cylindrical pore. The results of earlier work indicated that the ALM hexamer showed a preference for the cylindrical pores over the toroidal pores [61], however, this subsequent MD study revealed that the ALM Q7K hexamer formed semitoroidal pores. These observations suggested that the charged residues located in the N-terminal part of peptides played an important role in the formation of toroidal pores. Although the configuration of pores formed by ALM Q7K analogue differed from that observed previously for ALM [61], regarding the transmembrane orientations of peptides, the helicity of ALM Q7K was found to be comparable to

that calculated for ALM. In a further study, MD simulations were carried out by *Mihajlovic* and *Lazaridis* [63], applying an implicit membrane model in order to investigate the binding of ALM to the flat membrane, as well as to the pores with different shapes. Based on the MD trajectories, effective binding energies of ALM were calculated either for its adsorbed state on flat membrane, or for its inserted states in the cylindrical and toroidal pores. The results indicated that the binding energy of ALM was more favorable in the case of pores as compared to that calculated in the case of flat membrane. Nevertheless, these energies revealed a similar binding of ALM to the cylindrical and toroidal pores, indicating that ALM did not show a preference for one of afore-mentioned two types of pores. An additional finding of this study was that the effective binding energies of different AMPs could be correlated with their hemolytic activities.

4. Recent Results about the Biological Activities of Alamethicin. – Data about the biological activities of ALM published since the appearence of the review by *Leitgeb et al.* [1] are summarized in Table 2.

4.1. *Antimicrobial Activities of Alamethicin.* As summarized by *Leitgeb et al.* [1], ALM was previously shown to be effective against fungi (*Blastomyces dermatitidis*, *Hormodendrum compactum*, *Histoplasma capsulatum*, *Trichophyton mentagrophytes*, *Coccidioides immitis*, *Yarrowia lipolytica* and *Saccharomyces cerevisiae*), mollicute parasites of humans, animals and plants (*Acholeplasma*, *Mycoplasma* and *Spiroplasma* species) and Gram-positive bacteria (e.g. *Streptococcus*, *Staphylococcus*, *Bacillus*, *Nocardia*, *Bacillus*, *Corynebacterium* species and anaerobic Gram-positive rumen bacteria), but – except from *Sinorhizobium meliloti* – ineffective against most of the Gram-negative bacteria

tested so far, as the outer membrane lipopolysaccharides are probably forming a strong diffusion barrier against peptaibols.

Fassi Fehri et al. [64] studied the activities of AMPs including ALM against *Mycoplasma pulmonis*, the agent of murine respiratory mycoplasmosis. The authors found that ALM displayed a minimal inhibitory concentration (MIC) of 6.25 μM and demonstrated its synergy with enrofloxacin. *Thippeswamy et al.* [65] studied the development of ALM resistance in *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus cereus* strains by studying biomimetic membranes prepared from total cellular lipid extracts of wild type and ALM resistant bacteria. IC_{50} concentrations of the wild type *S. aureus*, *E. faecalis* and *B. cereus* strains were 5.5, 3.25 and 2.0 μg/ml, while resistant variants had IC_{50} values of 29.0, 17.0 and 9.5 μg/ml, respectively. As revealed by phospholipid profile analysis, the ratio of amino-group containing phospholipids to amino-group lacking ones increased in resistant variants of *S. aureus* and *B. cereus* but decreased in resistant variants of *E. faecalis* relative to the wild type strains. Linoleic acid could be detected only in resistant *B. cereus*. Acquisition of resistance to ALM was accompanied by a decrease in ALM insertion in the biomimetic membrane as well as changes in membrane fluidity and surface charge. *Mehla* and *Sood* [66] selected and characterized variants of *E. faecalis* that are resistant to different doses of ALM in order to further study the mechanisms of ALM resistance. The development of dose dependent resistance could be observed. The authors suggested that the formation of bacterial cell aggregates – which was observed in resistant cells – could be the primary resistance mechanism, as the cell surface exposed to ALM is smaller in aggregated cells. The increased membrane rigidity due to altered fatty acids in the case of ALM resistant variants was correlated with limited membrane penetration by ALM. *Ayers et al.* [67] examined the

antibacterial activities of peptaibols including ALM F50 and ALM II from two unidentified fungi of the order *Hypocreale*s against *S. aureus* and methicillin-resistant *S. aureus* (MRSA). MIC values of ALM F50 and ALM II were 35 and 12 μg /ml, respectively towards *S. aureus*, while 140 and 23 μg /ml, respectively towards MRSA.

During a study on the induction of a non-specific permeability transition in mitochondria of the yeasts *Yarrowia lipolytica* and *Dipodascus (Endomyces) magnusii*, *Kovaleva et al.* [68] reported that ALM induced large-amplitude swelling of mitochondria and the formation of 1 nm channels in mitochondrial membranes permeable to divalent cations and low-molecular weight compounds. *Kuharczyk et al.* [69] studied the pathogenesis of human mtDNA mutations using a *S. cerevisiae* model where they used ALM to permeabilize mitochondria.

A recent study shed light on the antiprotozoan activity of ALM. *Ishiyama et al.* [70] performed a wide-scale screen for antitrypanosomal compounds. Besides leucinostatin (A and B) and tsushimycin, ALM I was also identified as a potential trypanocidal compound as it showed antitrypanosomal activities both *in vitro* and *in vivo*. ALM I showed a potent impact against the examined *T. brucei brucei* strain GUTat 3.1 and *T. brucei rhodesiense* strain STIB900. In order to be able to evaluate both antitrypanosomal and cytotoxic activity, the authors introduced a selectivity index (SI: IC_{50} of cytotoxicity divided by IC_{50} of antitrypanosomal activity). Similarly to the other two peptides tested, ALM had a higher and lower SI than suramin in the case of the examined *T. b. brucei* and *T. b. rhodesiense* strain, respectively. Unlike leucinostatin B at 1.0 mg/kg×4 and tsushimycin at 50 mg/kg×4, ALM I did not achieve a cure at a dose of 3.0 mg/kg×4 in the *T. b. brucei* S427 acute mouse model, however, it extended the mean of survival days: ALM-treated animals

survived approximately threefold longer than the untreated controls. The mode of action of ALM and the two other tested peptide antibiotics was suggested to be via interaction with the membrane-lipid layer of trypanosomes [70].

4.2. *Effects of Alamethicin on Plants.* ALM effects on plants already known before the publication of the review by *Leitgeb et al.* [1] include emission of methylsalicylate as well as induction of benzoic acid and salicylic acid in *Arabidopsis thaliana*, emission of volatiles (methyl salicylate, 4,8-dimethylnona-1,3,7-triene, 4,8,12-trimethyltrideca-1,3,7,11-tetraene) and upregulation of salicylate and jasmonate biosynthesis in lima bean (*Phaseolus lunatus*), induction of terpene synthase expression in *Lotus japonicus*, elicitation of tendril coiling in pea (*Pisum sativum*), bryony (*Bryonia dioica*) and *Lathyrus* spp., as well as permeabilization of plasma and mitochondrial membranes and causing cell death at high concentrations in the case of tobacco (*Nicotiana tabacum*) [1].

In the study of *Lühring et al.* [71], the tonoplast of the giant green alga *Chara corallina* proved to be a simple but adequate membrane to explore the formation of new membrane pores by exogenously applied ALM: the first Cs^+-conducting pores could be recorded within about 30 s of ALM administration at a concentration of 11 μM.

Juszczuk et al. [72] measured the respiratory activities of isolated wild type and deletion mutant mitochondria of cucumber (*Cucumis sativus*) in the presence and absence of ALM. Addition of ALM increased oxygen consumption with NADH both in the leaf and root mitochondria of the wild type. In leaf mitochondria, the addition of ALM during malate oxidation and complete access of substrates and cofactors increased the respiration rates by ~40% in both the wild type and the mutant MSC16 (which is characterized by slower growth and leaf spots of mosaic type). In root

mitochondria, the increase in malate oxidation in the presence of ALM was 60 and 70% in wild type and MSC16 mitochondria, respectively [72].

Rippa et al. [73] examined the death process in *A. thaliana* treated with ALM. ALM triggered rapid death with 100% mortality at a concentration of 50 μM: the rigidity of the leaves was lost and they became soft already 1 h after the treatment. This effect of ALM proved to be associated with the dramatic loss of intact RNA due to the degradation of 18S and 25S ribosomal RNA and in the inhibition of protein synthesis. Based on their results, the authors suggested that a specific stimulus activates RNases which leads to the cleavage of cytosolic and chloroplastic rRNA molecules at specific sites. Experiments conducted with ALM-dUL, a synthetic ALM analogue in which all Aib residues are replaced by leucine moieties, showed that the Aib residues are essential for the induction of rRNA cleavage and triggering plant death [73]. In a subsequent study [74], the cell death induced in *A. thaliana* cell cultures by high concentrations of ALM was found to be associated with cell shrinkage and DNA fragmentation which are characteristic to programmed cell death, while the lesion development in mature plants induced by lower ALM-concentrations was characterized as a hypersensitive-like response with callose deposition, production of reactive oxygen species (ROS) and phenolic compounds as well as the transcription of defense-related genes. The authors compared the effect of ALM with melittin, a natural antimicrobial from the venom of the bee *Apis mellifera* as well as with the peptaibol ampullosporin A and synthetic analogues of the peptaibols cervinin and trichogin, and found that the response amplitude in *A. thaliana* increased with the peptaibol's membrane permeabilization ability and was strongly associated with α-aminoisobutyric acid content.

In the case of *A. thaliana* leaves, the transcription of geranyllinalool synthase was found to be upregulated after treatment with ALM, resulting in induced emission of (E,E)-geranyllinalool and its suggested degradation product 4,8,12-trimethyltrideca-1,3,7,11-tetraene, which is emitted also after herbivore attack and is assumed to play a role in attracting predators or parasitoids of herbivores [75]. *Bruinsma et al.* [76] used ALM to induce an early step of plant defence signal transduction in Brussels sprouts plants (*Brassica oleracea* var. *gemmifera*), and to compare its effect with that of jasmonic acid (affecting a late step of plant defence) on volatile emission and on the behavioural response of females of the white butterfly parasitoid wasp *Cotesia glomerata* to volatiles. The four major volatile components emitted were the monoterpenes limonene, 1,8-cineole, sabinene and α-thujene. *C. glomerata* was attracted to plants treated with ALM in a dose-dependent manner. ALM proved to be a 20 times more potent inducer of indirect plant defence than jasmonic acid [76].

In order to enable the *in situ* investigation of callose synthesis regulation, *Aidemark et al.* [77] applied ALM for the permeabilization of *A. thaliana* and tobacco cell suspensions. ALM was shown to permeabilize the plasma membrane, the envelope of plastids and the inner mitochondrial membrane, making the studying of the regulation of plasma membrane-bound callose synthesis possible. Plastids were permeabilized at somewhat higher ALM concentrations than the plasma membrane. The results of this study suggested that ALM-induced permeabilization mimics the signal for the induction of a defense response against plasmodesmal leakage, which leads to the closing of plasmodesmata, assisted by callose formation being induced by the elevated Ca^{2+}.

In a subsequent study, *Aidemark et al.* [78] pre-treated tobacco (*Nicotiana tabacum* L. cv Bright Yellow-2) cells with elicitors of defence responses in order to study whether this affects permeabilization by ALM. It turned out from oxygen consumption experiments that added *Trichoderma* cellulase induced cellular resistance to ALM, which was independent of novel protein synthesis and proved to be associated with changes in the plasma membrane lipid composition of tobacco cells: isolated plasma membranes from cellulase-pretreated tobacco cells contained less negatively charged phospholipids than the control membranes and could be characterized with higher ratios of membrane lipid fatty acid to protein and sterol [78].

Maischak et al. [79] tested ALM application-elicited electrical signals on lima bean (*P. lunatus*) and barley (*Hordeum vulgare*) using the non-invasive method with microelectrodes placed in the apoplasm of the sub-stomatal cavities of open stomata. The authors demonstrated that both local and long-distance electrical signals can be induced by treatment with ALM. In the case of lima bean, a 40 mV depolarization was induced and detected at a distance of 5 cm on the same leaf upon a 5 nM ALM stimulus. In the case of barley, the application of 25 nM ALM on one leaf resulted in a 45 mV depolarization response on a different leaf at a distance of 25 cm, indicating that ALM induced a systemic electrical response [79].

In the study of *Ng et al.* [80], both miR163 – a recently evolved miRNA in *A. thaliana* – and its target genes PXMT1 and FAMT – encoding a family of small molecule methyltransferases involved in secondary metabolite biosynthetic pathways – proved to be induced by ALM treatments, suggesting their potential roles in defense response pathways.

In a recent study on the spikemoss *Selaginella moellendorfii*, *Li et al.* [81] found that while untreated plants did not emit terpenes, a number of monoterpenes including linalool and sesquiterpenes (e.g. β-elemene, germacrene D, β-sesquiphellandrene, and nerolidol) could be detected from plants treated with ALM in order to mimick pathogen infection. Furthermore, some *S. moellendorfii* microbial terpene synthase-like genes were shown to be induced by ALM treatment, suggesting that the emission of terpenoids may have a role in plant defense.

4.3. *Effects of Alamethicin on Animal Cells.* Regarding the effects on arthropods, ALM was found to induce tissue damage in *Culex pipiens* mosquito larvae, induced rapid efflux of intracellular K^+ through the plasma membrane of the fall armyworm *Spodoptera frugiperda* and the spruce budworm *Choristoneura fumiferana*, and proved to be toxic to the brine shrimp *Artemia salina* and the water flea *Daphnia magna* [1]. In a recent study, ALM was shown to cause maximal swelling of the mitochondria in the case of brown shrimp (*Crangon crangon*) and common prawn (*Palaemon serratus*) [82].

Poirier et al. [83] performed embryotoxicity bioassays with oysters (*Crassostrea gigas*) in order to assess the acute toxicity of ALM as a reference compound and different groups of peptaibols produced by a marine strain of *T. longibrachiatum* isolated from mussels (*Mytilus edulis*). ALM showed 22±4% abnormalities (segmented eggs, normal or malformed embryos, D-larvae with convex hinge, indented shell margins, incomplete shell or protruded mantle) at the lowest tested concentration of 0.5 nM, which increased to 64±38% at 23 nM. A further increase in the concentration did not increase the ratio of abnormalities. An EC_{50} value of 31±3 nM could be determined for the ALM mixture (Sigma–Aldrich, A4665) consisting of analogues F50/5, F50/6a, F50/7 and F50/8b [83].

During the examination of the antihelminthic activities of peptaibols including ALM F50 against Barber's pole worm (*Haemonchus contortus*), the IC_{50} values (determined as the concentration reducing cellular proliferation by 50% relative to untreated controls following 72 h of continuous exposure) proved to be 0.2 μg/ml for ALM F50 [67].

The review of *Leitgeb et al.* [1] discussed previous results about the effects of ALM on mammalian cells, including oral toxicity towards mice at high concentrations, cytolytic activity towards *Ehrlich* ascite tumor cells, rat peritoneal mast cells as well as bovine and mouse lymphocytes, hemolytic activity towards human erythrocytes, induction of spontaneous permeabilization of plasma membrane to ATP in mouse lymphocytes, motility inhibition, plasma-membrane damage, depletion of intracellular esterases and mitochondrial depolarization in boar spermatozoa, membrane-modifying activity and dissipation of mitochondrial membrane potential in human lung epithelial carcinoma cells, feline fetus lung cells and murine neuroblastoma cells, catecholamine release in cat adrenal glands as well as permeabilization of cells to Ca^{2+}, Mn^{2+}, and Ni^{2+} in bovine adrenal chromaffin cells [1].

Ayers et al. [67] examined the cytotoxicity of peptaibols including ALM F50 and ALM II from two unidentified fungi of the order Hypocreales towards human breast carcinoma (MCF-7), human large cell lung carcinoma (H460), human astrocytoma (SF-268), fibroblast (IMR90) and human melanoma (MDA-MB-435) cell lines, and determined the IC_{50} values (concentrations required to reduce cellular proliferation by 50% relative to untreated controls following 72 h of continuous exposure) of 2.2, 3.4, 2.3, 4.8 and 8.9 μM for ALM F50 and 1.1, 1.6, 1.7, 4.0 and 6.2 μM for ALM II, respectively.

ALM can be used to permeabilize membranes of different mammalian cell types. It is frequently used to permeabilize the cytoplasma membrane [84] and membranes of the endoplasmatic reticulum [84a], vesicles [85] or mitochondria [86]. *Weidema et al.* [84b] studied the permeabilization of the cell membrane of neuron-like rat pheochromocytoma cells (PC12) by zervamicin IIB – a 16-mer peptaibol capable of producing voltage-dependent conductances in artificial membranes – with the whole-cell patch-clamp technique and compared it with the permeabilization of ALM F50/5 and the cation channel-forming peptide antibiotic gramicidin D. The permeabilizing effects of ALM and zervamicin IIB proved to be different from those of gramicidin D, they were increased by cis-positive membrane potentials in the physiological range and may include calcium entry into the PC12 cell. *Doleh* and *Romani* [87] applied ALM to enhance permeability of the endoplasmic reticulum (ER) membrane in hepatocytes of male Sprague–Dawley rats to glucose-6-phosphate bypassing the transport mechanism, and found that the addition of ALM to the incubation system results in a marked decrease in the Mg^{2+} concentration present within the ER lumen. ALM is also routinely used to form pores in liver microsomes [88] to activate UDP-glucuronosyltransferases and predict glucuronidation clearance for drugs.

During the past five years, ALM (Sigma–Aldrich, A4665) has been used as a reference compound during the description of the biological activities of newly described peptaibols towards mammalian cells [89] and in studies aimed at the investigation of mitochondrial functions.

Using ALM as a reference compound during the study of acrebol, a peptaibol isolated from the filamentous fungus *Acremonium exuviarum*, *Andersson et al.* [89a] found that ALM depleted boar spermatozoa from ATP and NADH at the same

exposure concentrations (EC_{50} = 150 ng dry substance/ml of extended boar semen) which inhibited motility, and destroyed the plasma membrane permeability barrier. At a concentration of 500 ng/ml, ALM was found to initiate fast cell death of MIN-6 mouse insulinoma cells observable by propidium iodide staining already after 4 h of incubation, and to induce a massive release of cytochrome c from the mitochondria of MIN-6 cells [90]. According to this study, ALM induced high amplitude swelling of isolated rat liver mitochondria in sucrose-mannitol-based media and liberated considerable amounts of cytochrome c from the mitochondria, which is a prerequisite of apoptosis. ALM was found to induce swelling in both isolated rat liver and non-synaptic rat brain mitochondria, the permeabilization of the outer mitochondrial membrane with ALM enabled the release of apoptosis-inducing factor [91].

ALM was used as a control for maximal cytochrome c release in the case of brain mitochondria of male Sprague-Dawley rats [92], mitochondria of mouse liver [93], human tumor cell lines [93a], dopaminergic neuronal MES cells and human neuroblastoma cells [94]. Similarly, *Lecoeur et al.* [95] used ALM as a standard of cytochrome c release detection in mitochondria isolated from liver, brain, and heart of mice. *Morota et al.* [96] used ALM to induce a standardized maximal swelling response when comparing the capacity of calcium retention in spinal cord and brain mitochondria of adult male Wistar rats. ALM was used as a positive control to demonstrate the rapid induction of mitochondrial swelling and Ca^{2+} release by *Perchellet et al.* [97] in the case of mitochondria isolated from mouse liver. ALM was also used by *Konrad et al.* [98] as a calibration standard causing maximal swelling of mitochondria. *Hansson et al.* [99] calculated the extent of swelling in the case of adult human brain and liver mitchondria as the calcium-induced decrease in light scattering compared to that by ALM.

Hansson et al. [100] studied the calcium-induced generation of ROS in brain mitochondria of adult male Wistar rats, and found that unspecific permeabilization of the inner mitochondrial membrane by ALM immediately decreased the respiratory rate and increased the level of ROS independently of calcium, which was further potentiated if NAD(H) was added to the system. *Kristian et al.* [101] studied calcium-induced precipitate formation in brain mitochondria and found that the addition of ALM after carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) induced essentially complete liberation of intramitochondrial calcium. *Gerencser et al.* [102] labeled mitochondria of rat cortical astrocytes and neurons by transient transfection with plasmid vectors encoding mitochondrially targeted DsRed2 (mito-DsRed2). Fluorescence images revealed that after treatment with ALM, tubular mitochondria of primary cortical astrocytes shortened in length and swelled in width into large spherical structures filled with mito-DsRed2. ALM treatment also led to the rupture of the inner mitochondrial membrane and to the DsRed2-release from swollen mitochondria. ALM-evoked swelling was only half or less than half as extensive in neurons as in astrocytes [102]. *Mirandola et al.* [103] found that the inhibitory effect of methylmalonate (but not that of malonate) on succinate-supported oxygen consumption of brain mitochondria was minimized after nonselective permeabilization of mitochondrial membranes by ALM. Permeabilization of isolated rat forebrain mitochondria with ALM abolished the reduction of oxygen consumption elicited by ethylmalonic acid, methylmalonic acid and butylmalonic acid in succinate maintained respiring mitochondria [104]. *Tretter* and *Adam-Vizi* [105] investigated the effect of high Ca^{2+} load in ALM-treated mitochondria from the brain of male guinea-pigs, and found that the treatment did not alter the effect of Ca^{2+}, the elevated H_2O_2 generation in response to 10 μM Ca^{2+}

was unaffected in mitochondria that were made functionally incompetent by ALM treatment.

Salnikov et al. [106] applied ALM for the perforation of mitochondrial membranes in cardiac mitochondria, as it is capable of forming giant pores through both mitochondrial membranes. The simple perforation of both membranes of isolated cardiac mitochondria with ALM at a concentration of 80 μg/ml resulted in swelling of the mitochondria and a significant increase in the density of intramitochondrial nanoparticles. To facilitate studies of pharmacological pore modulation of the mitochondrial permeability transition pore (a voltage-dependent, large-conductance channel of the inner membrane playing an important role in different pathophysiological conditions), *Christensen et al.* [107] developed an assay using neonatal cardiomyocytes, where ALM was used to mediate dissipation of mitochondrial membrane potential. In another study aimed at the identification of the sources of H_2O_2 production by rat heart mitochondria, the addition of ALM resulted in 3-fold stimulation of the decomposition of H_2O_2 by intramitochondrial catalase [108]. *Kaasik et al.* [109] used the measurement of passive force developed by saponin-permeabilized mouse ventricular fibres as a sensor for compression of the myofibrils. The authors confirmed the mitochondrial swelling effect of ALM in cardiomyocytes (more homogenous mitochondrial mass, sometimes without clear boundaries between individual mitochondria), and found that mitochondrial swelling induced by ALM markedly augmented mitochondrial size, resulted in drastic modification of nuclear shape, and that the incubation of permeabilized fibres with ALM significantly increased passive force.

5. Conclusions. – Due to extensive research efforts in different fields of biosciences, the amount of information available about ALM is extensively growing in the literature.

In spite of the fact that a large amount of experimental data was recorded, the organization of ALM pores in lipid membranes and their regulation are not completely understood presently and need further research activities. To help the examination of ALM incorporated into different lipid bilayers, a number of membrane-forming techniques were introduced and will be developed expectedly in the future, which allow the long-term investigations or high-throughput screening of potential ion channel peptides. The continued study of pore modulation effects and the electrical (conductance) features of membranes depending on the size variation of formed pores (or 'defects') also has a great importance and can enable the application of the lipid-ALM system in the biosensors as bionanoelectronic or biomechanotransductive interfaces.

Recent X-ray diffraction, spectroscopic and theoretical studies provided new valuable results with regard to the structural and conformational properties of ALM and its analogues, as well as to the 3D structure of ALM channels. These structural investigations, applying several novel experimental techniques and computational methods, supplied more information not only about the structural and dynamic features of ALM peptides and their channels, but also about the interactions evolved between ALM peptides and a variety of lipid bilayers. On the whole, the recent experimental and theoretical studies led to numerous relevant observations, which might provide deeper insights into the mechanism of action of ALM at the molecular level.

Publications of the past five years revealed further data about the antibacterial and antifungal activities of ALM as well as about ALM resistance in microbes. New data were reported about its antiprotozoan activity, details about its role in induction of electrical signals, defence responses and cell death in different plants, about its effects on further animal organisms like oysters and nematodes as well as on a series of different mammalian cell cultures. ALM is also more and more routinely applied for the permeabilization of biological membranes, furthermore, as a model compound of the family of peptaibols, today it is an indispensable reference substance in the intensively growing field of peptaibol research. Moreover, based on its diverse biological acivities it can be expected that further application possibilities of ALM itself may be suggested in the near future.

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REFERENCES

[1] B. Leitgeb, A. Szekeres, L. Manczinger, C. Vágvölgyi, L. Kredics, *Chem. Biodivers.* **2007**, *4*, 1027.

[2] T. Degenkolb, J. Kirschbaum, H. Brückner, *Chem. Biodivers.* **2007**, *4*, 1052.

[3] C. E. Meyer, R. Reusser, *Experientia* **1967**, *23*, 85.

[4] T. Degenkolb, R. Dieckmann, K. F. Nielsen, T. Gräfenhan, C. Theis, D. Zafari, P. Chaverri, A. Ismaiel, H. Brückner, H. von Döhren, U. Thrane, O. Petrini, G. J. Samuels, **2008**, *Mycol. Progr.* **7**, 177.

[5] T. Degenkolb, H. Brückner, *Chem. Biodivers.* **2008,** *5,* 1817.

[6] T. Degenkolb, T. Gräfenhan, H. I. Nirenberg, W. Gams, H. Brückner, *J. Agric. Food Chem*. **2006,** *54,* 7047.

[7] M. A. Marahiel, T. Stachelhaus, H. D. *Mootz, Chem. Rev.* **1997**, *97*, 2651.

[8] B. Manavalan, S. K. Murugapiran, G. Lee, S. Choi, *BMC Structural Biology* **2010**, *10,* 1.

[9] H. Duclohier,H. Wroblewski, *J. Membr. Biol.* **2001**, *184*, 1.

[10] M. Ashrafuzzaman, O. S. Andersen, R. N. McElhaney*, Biochim. Biophys. Acta* **2008**, *1778,* 2814.

[11] M. Ashrafuzzaman, M. Duszyk, J. A. Tuszynski, *J. Phys.: Conf. Ser.* **2011,** *329*, 012029.

[12] M. Ashrafuzzaman, C.-Y. Tseng, M. Duszyk, J. A. *Chem. Biol. Drug Design* **2012**, *80,* 992.

[13] P. Wadhwani, J. Reichert , J. Bürck, A. S. Ulrich, *Eur. Biophys. J*. **2012**, *41,* 177.

[14] B. A. Heitz, I. W. Jones, H. K. Hall Jr., C. A. Aspinwall, S. S. Saavedra, *J. Am. Chem. Soc*. **2010**, *132,* 7086.

[15] T.-J. Jeon, N. Malmstadt, J. L. Poulos, J. J. Schmidt, *Biointerphases* **2008***, 3,* 96.

[16] L. M. Harriss, B. Cronin, J. R. Thompson, M. I. Wallace, *J. Am. Chem. Soc.* **2011**, *133*, 14507.

[17] J. A. Maurer, V. E. White, D. A. Dougherty, J. L. Nadeau, *Biosens. Bioelectron*. **2007**, *22,* 2577.

[18] J. L. Poulos, T.-J. Jeon, R. Damoiseaux, E. J. Gillespie, K. A. Bradley, J. J. Schmidt, *Biosens. Bioelectron.* **2009**, *24*, 1806.

[19] A. J. Krauson, J. He, W. C. Wimley, *Biochim. Biophys. Acta* **2012**, *1818*, 1625.

[20] H. Duclohier, H. Wróblewski, *J. Membr. Biol.* **2001**, *184*, 1.

[21] R. Chiriac, T. Luchian, *Biophys. Chem.* **2007**, *130*, 139.

[22] R. Chiriac, T. Luchian, *J. Membr. Biol.* **2008**, *224*, 45.

[23] L. Mereuta, R. Chiriac, T. Luchian, *J. Optoelectron Adv. Mater.* 2008, *10,* 1837.

[24] A. Apetrei, L. Mereuta, T. Luchian, *Biochim. Biophys. Acta*, **2009**, *1790,* 809.

[25] L. Mereuta, A. Asandei, T. Luchian, *PLoS ONE* **2011**, *6,* 25276.

[26] D. Aliverdieva, D. Mamaev, L. Snezhkova, C. Sholtz, *Toxicol. in Vitro* **2012**, *26*, 939.

[27] N. Vedovato, G. Rispoli, *Eur. Biophys. J*. **2007**, *36,* 771.

[28] T. Katsu, T. Imamura, K. Komagoe, K. Masuda, T. Mizushima, *Anal. Sci*. **2007**, *23,* 517.

[29] V. Oliynyk, U. Kaatze, T. Heimburg, *Biochim. Biophys. Acta* **2007**, *1768,* 236.

[30] V. Oliynyk, M. Jäger, T. Heimburg, V. Buckin, U. Kaatze, *Biophys. Chem*. **2008**, *134,* 168.

[31] G. Pabst, S. Danner, R. Podgornik, J. Katsaras, *Am. Chem. Soc*. **2007**, *10,* 1021.

[32] P. Wessman, M. Morin, K. Reijmar, K. Edwards, *J. Coll. Int. Sci.* **2010**, *346*, 127.

[33] K. Bertelsen, B. Vad, E. H. Nielsen, S. K. Hansen, T. Skrydstrup, D. E. Otzen, T. Vosegaard, N. Chr. Nielsen, J. *Phys. Chem.* **2011**, *115*, 1767

[34] M. Mayer, V. Semetey, I. Gitlin, J. Yang, G. M. Whitesides, *J. Am. Chem. Soc*. **2008**, *130,* 1453.

[35] N. Misra, J. A. Martinez, S.-C. J. Huang, Y. Wang, P. Stroeve, C. P. Grigoropoulos, A. Noy, *Proc. Natl. Acad. Sci. USA* **2009**, *106,* 13780.

[36] D. Marsh, Biophys. J. **2007**,*93*, 3884.

[37] E. Stava, M. Yu, H. C. Shin, H. Shin, J. Rodriguez, R. H. Blick, *Lab Chip*, **2012**, *12,* 80.

[38] T. N. Kropacheva, J. Raap, *Chem. Biodivers*. **2007**, *4,* 1388.

[39] M. Crisma, C. Peggion, C. Baldini, E. J. MacLean, N. Vedovato, G. Rispoli, C. Toniolo, *Angew. Chem. Int. Ed*. **2007**, *46*, 2047.

[40] R. O. Fox, F. M. Richards, *Nature* **1982**, *300,* 325.

[41] S. Qian, W. Wang, L. Yang, H. W. Huang, *Biophys. J.* **2008**, *94*, 3512.

[42] E. S. Salnikov, H. Friedrich, X. Li, P. Bertani, S. Reissmann, C. Hertweck, J. D. J. O'Neil, J. Raap, B. Bechinger*, Biophys. J*. **2009**, *96*, 86.

[43] K. Bertelsen, J. M. Pedersen, B. S. Rasmussen, T. Skrydstrup, N. C. Nielsen, T. Vosegaard, *J. Am. Chem. Soc.* **2007**, *129,* 14717.

[44] K. Bertelsen, B. Paaske, L. Thøgersen, E. Tajkhorshid, B. Schiøtt, T. Skrydstrup, N. C. Nielsen, T. Vosegaard *J. Am. Chem. Soc*. **2009**, *131*, 18335.

[45] L. Thogersen, B. Schiřtt, T. Vosegaard, N. C. Nielsen, E. Tajkhorshid, *Biophys. J*. **2008**, *95,* 4337.

[46] A. Kouzayha, M. N. Nasir, R. Buchet, O. Wattraint, C. Sarazin, F. Besson, *J. Phys. Chem. B* **2009,** *113,* 7012.

[47] S. Ye, K. T. Nguyen, Z. Chen, *J. Phys. Chem. B* **2010**, *114,* 3334.

[48] S. Ye, H. Li, F. Wei, J. Jasensky, A. P. Boughton, P. Yang, Z. Chen, *J. Am. Chem. Soc*. **2012**, *134,* 6237.

[49] L. Stella, M. Burattini, C. Mazzuca, A. Palleschi, M. Venanzi, I. Coin, C. Peggion, C. Toniolo, B. Pispisa, *Chem. Biodivers*. **2007**, *4*, 1299.

[50] C. Peggion, M. Jost, W. M. De Borggraeve, M. Crisma, F. Formaggio, C. Toniolo, *Chem. Biodivers*. **2007**, *4*, 1256.

[51] A. D. Milov, R. I. Samoilova, Y. D. Tsvetkov, F. Formaggio, C. Toniolo, J. Raap, *J. Am. Chem. Soc.* **2007**, *129,* 9260.

[52] A. D. Milov, R. I. Samoilova, Y. D. Tsvetkov, M. De Zotti, C. Toniolo, J. Raap, *J. Phys. Chem. B Letters* **2008**, *112,* 13469.

[53] R. A. Jose, M. De Zotti, C. Peggion, F. Formaggio, C. Toniolo, W. M. De Borggraevea, *J. Pept. Sci.* **2011**, *17*, 377.

[54] D. Maisch, P. Wadhwani, S. Afonin, C. Böttcher, B. Koksch, A. S. Ulrich, *J. Am. Chem. Soc.* **2009**, *131*, 15596.

[55] D. Noshiro, K. Asami, S. Futaki, *Bioorg. Med. Chem*. **2012**, *10,* 1016.

[56] C. U. Hjørringgaard, B. S. Vad, V. V. Matchkov, S. B. Nielsen, T. Vosegaard, N. C. Nielsen, D. E. Otzen, T. Skrydstrup, *J. Phys. Chem. B* **2012**, *116*, 7652.

[57] J. Dittmer, L. Thøgersen, J. Underhaug, K. Bertelsen, T. Vosegaard, J. M. Pedersen, B. Schiøtt, E. Tajkhorshid, T. Skrydstrup, N. C. Nielsen, *J. Phys. Chem. B* **2009,** *113,* 6928.

[58] A. D. Milov, M. I. Samoilova, Y. D. Tsvetkov, M. Jost, C. Peggion, F. Formaggio, M. Crisma, C. Toniolo, J. Handgraaf, J. Raap, *Chem. Biodivers*. **2007**, *4*, 1275.

[59] A. D. Milov, R. I. Samoilova, Y. D. Tsvetkov, M. De Zotti, F. Formaggio, C. Toniolo, J. Handgraaf, J. Raap, *Biophys. J*. **2009**, *96*, 3197.

[60] H. Zhan, T. Lazaridis, *Biophys. Chem.* **2012**, *161,* 1.

[61] M. Mihajlovic, T. Lazaridis, *Biochim. Biophys. Acta* **2010**, *1798,* 1485.

[62] M. Mihajlovic, T. Lazaridis, *Biochim. Biophys. Acta* **2012**, *1818,* 1274.

[63] M. Mihajlovic, T. Lazaridis, *Biochim. Biophys. Acta* **2010**, *1798,* 1494.

[64] L. F. Fehri, H. Wróblewski, A. Blanchard, *Antimicrob. Agents. Chemother.* **2007**, *51,* 468.

[65] H. S. Thippeswamy, S. K. Sood, R. Venkateswarlu, I. Raj, *Ann. Microbiol*. 2009, **59,** 593.

[66] J. Mehla, S. K. Sood, *Appl. Environ. Microbiol*. **2011**, 786.

[67] S. Ayers, B. M. Ehrmann, A. F. Adcock, D. J. Kroll, E. J. Carcache de Blanco, Qi Shen, S. M. Swanson, J. O. Falkinham, M. C. Wani, S. M. Mitchell, C. J. Pearceh, N. H. Oberliesa, *J. Pept. Sci*. **2012**, *18,* 500.

[68] M. V. Kovaleva, E. I. Sukhanova, T. A. Trendeleva, M. V. Zylkova, L. A. Uralskaya, K. M. Popova, N.-E. L. Saris, R. A. Zvyagilskaya, *J. Bioenerg. Biomembr*. **2009**, *41,* 239.

[69] R. Kucharczyk, M-F. Giraud, D. Brèthes, M. Wysocka-Kapcinska, N. Ezkurdia, B. Salin, J. Velours, N. Camougrand, F. Haraux, J-P. Rago, *Int. J. Biochem. Cell Biol*. **2012** *in press,* DOI: 10.1016/j.biocel.2012.07.001

[70] A. Ishiyama, K. Otoguro, M. Iwatsuki, M. Namatame, A. Nishihara, K. Nonaka, Y. Kinoshita, Y. Takahashi, R. Masuma, K. Shiomi, H. Yamada, S. Omura, *J. Antibiot*. **2009**, *62*, 303.

[71] H. Lühring, V. D. Nguyen, L. Schmidt, U. S. R. Röse, *FEBS Lett*. **2007**, *581*, 5361.

[72] I. M. Juszczuk, J. Flexas, Bozena Szal, Zofia Dabrowska, M. Ribas-Carbo, A. M. Rychter, *Phys. Planta*. **2007**, *131,* 527.

[73] S. Rippa, H. Adenier, M. Derbaly, L. Beven, *Chem. Biodivers*. **2007**, *4*, 1360.

[74] S. Rippa, M. Eid, F. Formaggio, C. Toniolo, L. Béven, *ChemBioChem* **2010**, *11,* 2042.

[75] M. Herde, K. Gartner, T. G. Köllner, B. Fode, W. Boland, J. Gershenzon, C. Gatz, D. Tholl, *Plant Cell,* **2008***, 20,* 1152.

[76] M. Bruinsma, B. Pang, R. Mumm, J. J. A. van Loon, M. Dicke, *J. Exp. Bot*. **2009**, *60,* 2589.

[77] M. Aidemark, C. Andersson, A. G. Rasmusson, S. Widell, *BMC Plant Biol.* **2009**, *9*, 27.

[78] M. Aidemark**,** H. Tjellström**,** A. S. Sandelius**,** H. Stålbrand**,** E. Andreasson**,** A. G. Rasmusson**,** S. Widell**,** *BMC Plant Biol.* **2010**, *10*, 274.

[79] H. Maischak, M. R. Zimmermann, H. H. Felle, W. Boland, A. Mithöfer*, Plant Signal. Behav*. **2010**, *5*, 988.

[80] D. W-K. Ng, C. Zhang, M. Miller, G. Palmer, M. Whiteley, D. Tholl, Z. J. Chen, *Plant Cell* **2011**, *23,* 1729.

[81] G. Li, T. G. Köllner, Y. Yin, Y. Jiang, H. Chen, Y Xu, J. Gershenzon, E. Pichersky, F. Chen, *Plant Biol*. **2012**, *109,* 14711.

[82] C. Konrad, G. Kiss, B. Torocsik, V. Adam-Vizi, C. Chinopoulos, *PLoS ONE*, **2012**, *7,* 39839.

[83] L. Poirier, F. Quiniou, N. Ruiz, M. Montagua, J. Amiard, Y. F. Pouchus, *Aquatic Toxicol*. **2007**, *83*, 254.

[84] a) K. Révész, A. Tüttő, É. Margittai, G. Bánhegyi, J. É. Magyar, J. Mandl, M. Csala, *Int. J. Biochem. Cell. Biol*. **2007**, *39,* 922; b) A. F. Weidema, T. N. Kropacheva, J. Raap, D. L. Ypey, *Chem. Biodivers*. **2007**, *4*, 1347; c) J. É. Magyar, A. Gamberucci, L. Konta, É. Margittai, J. Mandl, G. Bánhegyi, A. Benedetti, M. Csala, *Int. J. Biochem. Cell Biol*. **2009**, *41,* 694; d) *S.* L. H. Zeiger, J. R. McKenzie, J. N. Stankowski, J. A. Martin, D. E. Cliffel, B. McLaughlin*, Biochim. Biophys. Acta* **2010**, *1802,* 1095.

[85] M. R. Montes, A. J. Spiaggi, J. L. E. Monti, F. Cornelius, C. Olesen, P. J. Garrahan, R. C. Rossi, *Biochim. Biophys. Acta* **2011**, *1808,* 316.

[86] a) P. Korge, P. Ping, J. N. Weiss, *Circ. Res*. **2008**, *103,* 873; b) T. Li, T. Brustovetsky, B. Antonsson, N. Brustovetsky, *Biochim. Biophys. Acta* **2008**, *1777,* 1409; b) A. M. Gusdon, T. V. Votyakova, C. E. Mathews, *J. Biol. Chem.* **2008**, *283*, 10690; c) B. Németi, Z. Gregus*, Toxicol. Appl. Pharmacol*. **2009**, *239,* 154

[87] L. Doleh, A. Romani, *Arch. Biochem. Biophys*. **2007**, *467,* 283.

[88] a) M. Chen, D. Howe, B. Leduc, S. Kerr, D. A. Williams, *Xenobiotica*, **2007**, *37,* 954; b) S. Kaivosaari, P. Toivonen, O. Aitio, J. Sipila, M. Koskinen, J. S. Salonen, M. Finel, *Drug Metab. Dispos*. **2008**, *36,* 1529; c) P. J. Kilford, R. Stringer, B. Sohal, J. B. Houston, A. Galetin, *Drug Metab. Dispos*. **2009**, *37*,

82; d) K. M. Knights, L. K. Winner, D. J. Elliot, K. Bowalgaha, J. O. Miners, *British J. Clin. Pharmacol.* **2009**, *68*, 402; e) H. E. Cubitt, J. B. Houston, A. Galetin, *Pharm. Res*. **2009**, *26,*1073; f) U. A. Argikar, J. Gomez, D. Ung, H. P. Parkman, S. Nagar*, Drug Metab. Dispos***. 2010**, *38,* 1295; g) C. S. Mazur, J. F. Kenneke, J. K. Hess-Wilson, J. C. Lipscomb*, Drug Metab. Dispos*. **2010**, *38*, 2232; h) F. Nakamori, Y. Naritomi, M. Furutani, F. Takamura, H. Miura, H. Murai, S. Terashita, T. Teramura, *Drug Metab. Pharmacokinet*. **2011**, *26,* 465; i) H. Nishimuta, K. Sato, M. Yabuki, S. Komuro*, Drug Metab. Pharmacokinet*. **2011**, *26,* 592; j) H. Zhang, A. Tolonen, T. Rousu, J. Hirvonen, M. Finel*, Drug Metabol. Dispos*. **2011**, *39,* 456; k) M.-E. F. Mohamed, R. F. Frye, *Drug Metab. Dispos.* **2011**, *39,* 1522; l) M. Dostalek, M. H. Court, S. Hazarika, F. Akhlaghi, *Drug Metab. Dispos*. **2011**, *39,* 448; m) R. L. Walsky, J. N. Bauman, K. Bourcier, G. Giddens, K. Lapham, A. Negahban, T. F. Ryder, R. S. Obach, R. Hyland, T. C. Goosen, *Drug Metab. Dispos*. **2012**, *40*, 1051-1065; n) X. Liu, A. DeJesus, Z. Cao, D. Vardeman, B. Giovanella, *Int. J. Mol. Sci.* **2012**, *13*, 5498; o) K. L. Gill, J. B. Houston, A. Galetin, *Drug Metab. Dispos*. **2012**, *40*, 825;

[89] a) M. A. Andersson, R. Mikkola, M. Raulio, L. Kredics, P. Maijala, M.S. Salkinoja-Salonen, *J. Appl. Microbiol.* **2009**, *106*, 909; b) R. Mikkola, M. A. Andersson, L. Kredics, P. A. Grigoriev, N. Sundell, M. S. Salkinoja-Salonen, *FEBS J*. **2012**, *279*, 4172.

[90] G. Kruglov, M. A. Andersson, R. Mikkola, M. Roivainen, L. Kredics, N. L. Saris, M. S. Salkinoja-Salonen, *Chem. Res. Toxicol.* **2009**, *22*, 565.

[91] A. Joshi, V. Bondada, J. W. Geddes, *Exp. Neurol*. **2009**, *218,* 221.

[92] a) T. Li, T. Brustovetsky, B. Antonsson, N. Brustovetsky, **2008,** *1777,* 1409; b) T. Brustovetsky, T. Li, Y. Yang, J. Zhang, B. Antonsson, N. Brustovetsky, *Biochim. Biophys. Acta*. **2010,** *1797,* 1795.

[93] a) N. Buron, M. Porceddu, M. Brabant, D. Desgué, C. Racoeur, M. Lassalle, C. Péchoux, P. Rustin, E. Jacotot, A. Borgne-Sanchez, *PLoS ONE* **2010,** *5,* 9924; b) M. Porceddu, N. Buron, C. Roussel, G. Labbe, B. Fromenty, A. Borgne-Sanchez, *Toxicol. Sci*. **2012,** *129,* 332.

[94] A. K. Berger, G. P. Cortese, K. D. Amodeo, A. Weihofen, A. Letai, M. J. LaVoie, *Human Mol. Genet.* **2009***, 18,* 4317.

[95] H. Lecoeur, A. Borgne-Sanchez, O. Chaloin, R. El-Khoury, M. Brabant, A. Langonné, M. Porceddu, J. Briere, N. Buron, D. Rebouillat, C. Péchoux, A. Deniaud, C. Brenner, J. P. Briand, S. Muller, P. Rustin, E. Jacotot, *Cell Death Dis.* **2012**, *15*, e282.

[96] S. Morota, M. J. Hansson, N. Ishii, Y. Kudo, E. Elmér, H. Uchino, *J Neurochem***. 2007**, *103*, 2066.

[97] E. M. Perchellet, Y. Wang, K. Lou, H. Zhao,S. K. Battina, D. H. Hua, J. H. Perchellet, *Anticancer Res*. **2007**, *27*, 3259.

[98] C. Konrad, G. Kiss, B. Torocsik, V. Adam-Vizi, C. Chinopoulos, *PLoS ONE*, **2012**, *7,* 39839.

[99] M. J. Hansson, S. Morota, L. Chen, N. Matsuyama, Y. Suzuki, S. Nakajima, T. Tanoue, A. Omi, F. Shibasaki, M. Shimazu, Y. Ikeda, H. Uchino, Eskil Elmér, *J. Neurotrauma* **2011**, *28*, 143.

[100] M. J. Hansson, R. Mansson, S. Morota, H. Uchino, T. Kallur, T. Sumi, N. Ishii, M. Shimazu, M. F. Keep, A. Jegorov, E. Elmér, *Free Radic. Biol. Med.* **2008**, *45*, 284.

[101] T. Kristian, N. B. Pivovarova, G. Fiskum, S. B. Andrews, *J. Neurochem*. **2007**, *102*, 1346.

[102] A. A. Gerencser, J. Doczi, B. Töröcsik, E. Bossy-Wetzel, V. Adam-Vizi, *Biophys. J*. **2008**, *95*, 2583.

[103] S. R. Mirandola, D. R. Melo, P. F. Schuck, G. C. Ferreira, M. Wajner, R. F. Castilho, *J. Inherit. Metab. Dis.* **2008***, 31,* 44.

[104] A. U. Amaral, C. Cecatto, E. N. B. Busanello, C. A. J. Ribeiro, D. R. Melo, G. Leipnitz, R. F. Castilho, M. Wajner*, Mol. Genet. Metab*. **2012**, *105*, 84.

[105] L. Tretter, V. Adam-Vizi, *Free Radic. Biol. Med*. **2012** *in press,* DOI: 10.1016/j.freeradbiomed.2012.09.029

[106] V. Salnikov, Y. O. Lukyánenko, C. A. Frederick, W. J. Lederer, V. Lukyánenko, *Biophys. J*. **2007**, *92*, 1058.

[107] M. L. M. Christensen, T. H. Braunstein, M. Treiman, *Anal. Biochem*. **2008**, *378*, 25.

[108] V. G. Grivennikova, A. V. Kareyeva, A. D. Vinogradov, *Biochim Biophys Acta***. 2010**, *1797,* 939.

[109] A. Kaasik, M. Kuum, F. Joubert, J. Wilding, R. Ventura-Clapier, V. Veksler, *Cardiovasc. Res.* **2010**, *87*, 83.

Table 1. *ALM Analogues Due to Substitution and/or Incorporation of Certain Residues* (variable residues are marked bold)

ALM analogues	**Sequences**	**Ref.**
[Glu(OMe)^{18,19}] ALM F50/5[a])	Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-**Glu(OMe)**-**Glu(OMe)**-Phol	[49]
[Glu(OMe)^{7,18,19}] ALM F50/5	Ac-Aib-Pro-Aib-Ala-Aib-Ala-**Glu(OMe)**-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-**Glu(OMe)**-**Glu(OMe)**-Phol	[49] [50]
Fmc-[Glu(OMe)^{7,18,19}] ALM F50/5[b])	**Fmc**-Aib-Pro-Aib-Ala-Aib-Ala-**Glu(OMe)**-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-**Glu(OMe)**-**Glu(OMe)**-Phol	[49]
[Glu(OMe)^{7,18,19}] ALM F50/5-Fmc	Ac-Aib-Pro-Aib-Ala-Aib-Ala-**Glu(OMe)**-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-**Glu(OMe)**-**Glu(OMe)**-Phol-**Fmc**	[49]
[TOAC^{1},Glu(OMe)^{7,18,19}] ALM F50/5[c])	Ac-**TOAC**-Pro-Aib-Ala-Aib-Ala-**Glu(OMe)**-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-**Glu(OMe)**-**Glu(OMe)**-Phol	[50] [53] [58] [59]
[TOAC^{8},Glu(OMe)^{7,18,19}] ALM F50/5	Ac-Aib-Pro-Aib-Ala-Aib-Ala-**Glu(OMe)**-**TOAC**-Val-Aib-Gly-	[50] [58] [59]

	Leu-Aib-Pro-Val-Aib-Aib- **Glu(OMe)**-**Glu(OMe)**-Phol	
[TOAC^{16},Glu(OMe)^{7,18,19}] ALM F50/5	Ac-Aib-Pro-Aib-Ala-Aib-Ala- **Glu(OMe)**-Aib-Val-Aib-Gly-Leu- Aib-Pro-Val-**TOAC**-Aib-**Glu(OMe)**- **Glu(OMe)**-Phol	[39] [50] [51] [58] [59]
[TOAC^{1,16},Glu(OMe)^{7,18,19}] ALM F50/5	Ac-**TOAC**-Pro-Aib-Ala Aib-Ala- **Glu(OMe)**-Aib-Val-Aib-Gly-Leu- Aib-Pro-Val-**TOAC**-Aib-**Glu(OMe)**- **Glu(OMe)**-Phol	[50] [52]

[(*R*)-CF_3-Ala^5] ALM F30/3[d])	Ac-Aib-Pro-Aib-Ala-**((*R*)-CF_3-Ala^5)**-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Phol	[54]
[(*S*)-CF_3-Ala^5] ALM F30/3	Ac-Aib-Pro-Aib-Ala-**((*S*)-CF_3-Ala^5)**-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Phol	[54]
[(*R*)-CF_3-Ala^{10}] ALM F30/3	Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-**((*R*)-CF_3-Ala^5)**-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Phol	[54]
[(*S*)-CF_3-Ala^{10}] ALM F30/3	Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-**((*S*)-CF_3-Ala^5)**-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Phol	[54]
[(*R*)-CF_3-Ala^{16}] ALM F30/3	Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-**((*R*)-CF_3-Ala^5)**-Aib-Gln-Gln-Phol	[54]
[(*S*)-CF_3-Ala^{16}] ALM F30/3	Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-**((*S*)-CF_3-Ala^5)**-Aib-Gln-Gln-Phol	[54]
HG-ALM	**H_2N-His-Gly**-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Phe-**$CONH_2$**	[54]
HG-[Leu]ALM	**H_2N-His-Gly**-**Leu**-Pro-**Leu**-Ala-**Leu**-Ala-Gln-**Leu**-Val-**Leu**-Gly-Leu-**Leu**-Pro-Val-**Leu**-**Leu**-Gln-Gln-Phe-	[54]

	$CONH_2$	
HG-[Nva]ALM[e])	**H_2N-His-Gly**-**Nva**-Pro-**Nva**-Ala-**Nva**-Ala-Gln-**Nva**-Val-**Nva**-Gly-Leu-**Nva**-Pro-Val-**Nva**-**Nva**-Gln-Gln-Phe-**$CONH_2$**	[55]
HG-[Nle]ALM[f])	**H_2N-His-Gly**-**Nle**-Pro-**Nle**-Ala-**Nle**-Ala-Gln-**Nle**-Val-**Nle**-Gly-Leu-**Nle**-Pro-Val-**Nle**-**Nle**-Gln-Gln-Phe-**$CONH_2$**	[55]
ALM Q7K	Ac-Aib-Pro-Aib-Ala-Aib-Ala-**Lys**-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Pheol	[62]

[a]) Ac=acetyl, Phol=phenylalaninol, Glu(OMe)=γ-methyl glutamate. [b]) Fmc=9*H*-fluoren-9-ylacetyl. [c]) TOAC=2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid. [d]) CF_3-Ala=trifluoromethylalanine. [e]) Nva=norvaline. [f]) Nle=norleucine.

Table 2. *Biological Effects of ALM Reported since the Publication of the Review by Leitgeb et al.* [1]

Target	Effect of ALM	Ref.
Bacteria		
Enterococcus faecalis	inhibition, development of resistance	[65] [66]
Staphylococcus aureus	inhibition, development of resistance	[65]
	inhibition	[67]
methicillin-resistant *Staphylococcus aureus* (MRSA)	inhibition	[67]
Bacillus cereus	inhibition, development of resistance	[65]
Mycoplasma pulmonis	inhibition, synergy with enrofloxacin	[64]
Fungi		
Yarrowia lipolytica, *Dipodascus (Endomyces) magnusii*	large-amplitude swelling of mitochondria	[68]
Saccharomyces cerevisiae atp6-W136R mutant	permeabilization of mitochondria	[69]
Protozoa		
T. brucei brucei, T. brucei rhodesiense	antitrypanosomal and cytotoxic activity	[70]
Plants		

Chara corallina	pore formation in tonoplast membrane	[71]
Cucumis sativus	increased respiration rate and malate oxidation in leaf and root mitochondria	[72]
Arabidopsis thaliana	rapid death, induction of rRNA cleavage	[73]
Arabidopsis thaliana	cell shrinkage, DNA fragmentation and cell death in cell cultures by high ALM concentrations	[74]
	hypersensitive-like response (callose deposition, production of reactive oxygen intermediates and phenolic compounds, transcription of defense genes) by lower ALM concentrations	
Arabidopsis thaliana	induction of miR163 and its target genes	[80]
Arabidopsis thaliana	induced emission of (E,E)-geranyllinalool and 4,8,12-trimethyltrideca-1,3,7,11-tetraene	[75]

Brassica oleracea var. *gemmifera*	volatile emission, attraction of the parasitoid wasp *Cotesia glomerata*	[76]
A. thaliana and *Nicotiana tabacum* cell suspensions	permeabilization of the plasma membrane, the envelope of plastids and the inner mitochondrial membrane	[77]
Phaseolus lunatus	induction of local electrical signals	[79]
Hordeum vulgare	induction of long-distance electrical signals	[79]
Selaginella moellendorfii	induction of microbial terpene synthase-like genes and the emission of mono- and sesquiterpenes	[81]
Molluscs		
Crassostrea gigas	embryotoxicity	[83]
Nematodes		
Haemonchus contortus	inhibition of larval motility	[67]
Arthropods		
Crangon crangon and *Palaemon serratus*	maximal swelling of mitochondria	[82]
Mammals		
human breast carcinoma, human large cell lung	cytotoxicity	[67]

carcinoma, human astrocytoma, fibroblast and human melanoma cell lines		
neuron-like rat pheochromocytoma cell	permeabilization of the cell membrane	[84b]
rat hepatocytes	enhancment of ER membrane permeability to glucose-6-phosphate, decreasing Mg^{2+} concentration present within the ER lumen	[87]
human liver microsomes	permeabilization of microsomes and activation of UDP-glucuronosyl-transferases	[88a, b, c, e, f, g, j, m]
human intestinal microsomes		[88b, e, i, j]
rat liver microsomes		[88g, h]
boar spermatozoa	motility inhibition, depletion from ATP and NADH	[89a]
isolated rat liver mitochondria	high amplitude swelling, liberation of considerable amounts of cytochrome c	[90]
rat spinal cord and brain mitochondria	induction of maximal swelling response	[96]
mouse liver mitochondria	rapid induction of swelling	[97]
adult human brain and liver mitchondria	swelling	[99]

mitochondria of rat cortical astrocytes and neurons	swelling, rupture of the inner mitochondrial membrane	[102]
cardiomyocytes	mitochondrial swelling, drastic modification of nuclear shape	[109]
cardiac mitochondria	swelling, perforation of mitochondrial membranes, increase in the density of intramitochondrial nanoparticles	[106]
mouse insulinoma cells	fast cell death, massive release of cytochrome c from mitochondria	[90]
rat brain cells	release of cytochrome c from mitochondria	[92]
mouse liver cells		[93]
human tumor cell lines		[93a]
dopaminergic neuronal MES cells, human neuroblastoma cells		[94]
mouse liver, brain and heart cells		[95]
non-synaptic rat brain mitochondria	permeabilization, release of apoptosis-inducing factor	[91]
rat brain mitochondria	decreasing of the respiratory rate, increasing of the level	[100]

	of reactive oxygene species	
brain mitochondria	complete liberation of intramitochondrial calcium	[101]
neonatal cardiomyocytes	dissipation of mitochondrial membrane potential	[107]
rat heart mitochondria	stimulation of the decomposition of H_2O_2 by intramitochondrial catalase	[108]