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Differences in Membrane Pore Formation by Peptaibols[‡]

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Abstract: The efficiencies of membrane pore formation by 14 naturally occurring peptaibols and two structurally modified ampullosporins were compared using an artificial bilayer membrane model. Major differences were found in the dependence on peptide sequences and the constituting amino acids. Alamethicin F-30, chrysospermins C/D, paracelsin and texenomycin A displayed higher activity by several orders of magnitude in comparison with smaller peptaibols containing <17 amino acids such as ampullosporins, trichofumins, bergofungins and cephaibols. Biological activities such as the induction of pigment formation by the fungus *Phoma destructiva* and long acting hypothermia and depression of locomotor activity in mice were correlated with moderate membrane permeabilization. No or weak membrane activities corresponded with biological inactivity. Highly membrane-active structures such as alamethicin F-30, chrysospermin C, texenomycin A and paracelsin A displayed antibiotic effects against the fungus and toxicity in mice. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptaibols; membrane pore formation; stimulation of fungal morphogenesis; hypothermia in mice; neuroleptic activity

INTRODUCTION

Peptaibols and related lipopeptaibols (peptaibiotics) form a growing family of fungal peptides characterized by a sequence of 5–20 amino acids [1]. Among them the presence of several α-amino isobutyric acid moieties (Aib) is a typical feature determining the helix-forming properties of these structures [1,2]. Moreover, the *N*-terminus of the peptide chain is acetylated and the *C*-terminus is reduced to an amino alcohol. The related lipopeptaibols contain a fatty acid with >6 carbon atoms bound as an amide to the *N*-terminus, and the *C*-terminus is substituted by basic groups such as amino alcohols, argininol or others.

The 15mer peptaibol ampullosporin A (**1**) was reported to induce morphogenesis of *Phoma destructiva* and pigment formation in surface cultures of this fungus [3]. This effect was correlated with the induction of long acting hypothermia and the reduction of spontaneous motor activity in mice after a single intraperitoneal administration, signalling neuroleptic activity [3]. In the search for naturally occurring biosynthetic congenors of ampullosporin A, semisynthesis provided a series of modified structures differing in the above biological activities from the parent compound [4,5]. Moreover, new peptaibols such as trichofumins [6] and some representatives of the cephaibol family [7] displayed biological effects comparable to ampullosporin A. However, others such as bergofungins [5,8], texenomycin A [5,9], paracelsin A [5] and alamethicin F-30 [5] were distinguishable either by inactivity or by higher toxicity in mice under the same conditions.

Permeabilization of biological membranes due to the formation of voltage-dependent (gated) pores has been suggested as the general mode of action of

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the peptaibol family [2,10,11]. However, in the past no comprehensive investigations have been reported comparing the membrane activities of different peptaibol structures under identical conditions. In this paper the relative increase is reported of membrane conductivity caused by different peptaibols using a black lipid membrane model. The observed membrane effects were discussed in relation to the biological activities as inducers of fungal pigment production (morphogenesis) and long acting hypothermia in mice.

MATERIALS AND METHODS

All peptaibols were used as pure compounds except for chrysospermin C containing 25% of the related chrysospermin D [12]. Ampullosporins A (**1**), B and D were isolated from *Sepedonium ampullosporum* [3,4], chrysospermin C (**10**) from *Apiocrea chrysosperma* [12], trichofumins A (**2**) and C (**3**) from *Trichoderma* sp. [6], bergofungins A (**4**) and B (**5**) from *Emericellopsis donezkii* [8,13], texenomycin A (**12**) from *Moszia lindtneri* [9] and cephaibols B (**6**), C (**7**) and E (**8**) from *Acremonium tubaki* [7], alamethicin F-30 (**9**) and paracelsin A (**11**) were prepared from fermentations of *Trichoderma* sp., desacetyltryptophanyl-ampullosporin A (**15**) and hexamethyl ampullosporin A (**16**) were obtained by chemical semisynthesis from ampullosporin A [13,14].

Membrane Measurements

The measuring device and method of formation of black lipid bilayer membranes (BLM) and the measurement of alterations of membrane conductance in the presence of different peptaibols was as reported [9,14,15]. Signal recording was carried out by a computerized device. The output signals filtered at 30 Hz were stored in a PC held disk via analog input of an AT-MIO-16X card (National Instruments) and analysed with Win EDR r. 205 (Windows Electrophysiology Disk Recorder) from Strathclyde Electrophysiology Software (Dempster, UK).

For comparison of the relative efficiency in BLM permeabilization the increase in electric conductance was measured in the presence of peptaibols. Black lipid membranes were formed by a solution of L-α-lecithin (Sigma, P-5638) in heptane. Peptaibols from 20 mg/ml stock solutions (in ethanol) were added to the measuring cell with stirring during 10 min into a 12 ml volume of 200 mM KCl to a final concentration of 0.5 μg peptaibol/ml. Thereafter a lipid bilayer membrane was formed. The formation of BLM was monitored on a PC screen where pulses of capacitance current appeared in response to rectangular shape voltage which was applied to the membrane. Constant-in-time amplitude of the pulses indicated that the formation of bilayer was complete. Subsequently rectangular voltage pulses were switched off and the membrane was left for 10 min at zero voltage to allow the peptaibols to equilibrate between the water solution and the membrane. Then 70 mV of DC voltage was switched to the membrane, and the membrane current started to increase in response to the formation of ion-conducting pathways such as pores or channels in the membrane. The magnitude of the membrane current at the end of a 10 min period with 70 mV DC was recorded and the corresponding specific conductance was calculated (Figure 2). Control experiments were carried out under the same conditions but the addition of peptaibols was omitted. The data shown in the graphs represented the mean value of five to eight experiments.

In the case of alamethicin F-30 and chrysospermin C (Figure 1) a concentration of 0.5 μg/ml appeared to be too high for reliable measurements. Due to the comparably huge efficiency of the membrane pore formation, the concentrations were reduced to 0.25 μg/ml in separate experiments so as to improve the membrane stability and to adapt the membrane conductance for the current-measuring set up used. Thereafter the conductance shown in Figure 2 was extrapolated to a value that could be expected at 0.5 μg/ml using $n = 6$ as the order of concentration dependence of the membrane conductance.

Biological Assays

Antimicrobial activities against the fungal strain *Phoma destructiva* and induction of pigment formation by this test organism were determined using the agar well diffusion assay [3,4,5,12]. To each agar well (9 mm diameter) 100 μg of peptaibols in 50 μl methanol was applied. After 5 days incubation of the inoculated agar plate the appearance of a brownish halo indicated fungal morphogenesis, or inhibition zones showed antibiotic activity. Induction of long acting hypothermia in mice was investigated measuring the body temperature with a thermistor probe in the rectum of the animal [3]. The colonic temperature was recorded at 0.5, 1, 3, 5, 8 24, 48 and

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Ampullosporin A (**1**) : **PS; HS**
Ac-Trp-Ala-Aib-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Gln-Leu-Aib-Gln-Leuol
Trichofumin A (**2**) : **PS;** weak; **HS,** weak
Ac-Val-Gln-Leu-Val-Aib-Pro-Leu-Leu-Aib-Pro-Leuol
Trichofumin C (**3**) : **PS; HS**
Ac-Val-Gln-Val-Aib-Gln-Gln-Leu-Leu-Pro-Leu-Aib-Pro-Leuol
Bergofungin A (**4**) : **PN; HN**
Ac-Val-Aib-Aib-Aib-Val-Gly-Leu-Aib-Aib-Hypro-Gln-Iva-Hypro-Aib-Pheol
Bergofungin B (**5**) : **PS;** weak**; HN**
Ac-Val-Aib-Aib-Aib-Val-Gly-Leu-Val-Aib-Hypro-Gln-Iva-Hypro-Aib-Pheol
Cephaibol B (**6**) : **PS; HS**
Ac-Phe-Aib-Aib-Aib-Iva-Gly-Leu-Iva-Aib-Hypro-Gln-Iva-Hypro-Aib-Pro-Pheol
Cephaibol C (**7**) : **PS; HS**
Ac-Phe-Aib-Aib-Aib-Aib-Gly-Leu-Iva-Aib-Hypro-Gln-Aib-Hypro-Aib-Pro-Pheol
Cephaibol E (**8**) : **PN; HN**
Ac-Phe-Aib-Aib-Aib-Aib-Gly-Leu-Aib-Aib-Hypro-Gln-Iva-Hypro-Aib-Pro-Pheol
Alamethicin F-30 (**9**) : **A, PS** (in 20 fold lower concentration); **HT**
Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol
Chrysospermin C (**10**): **A, PS** (in 20 fold lower concentration); **HT**
Ac-Phe-Aib-Ser-Aib-Iva-Leu-Gln-Gly-Aib-Aib-Ala-Ala-Aib-Pro-Aib-Aib-Aib-Gln-Trpol
Paracelsin A (**11**) : **A, PN; HT**
Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol
Texenomycin A (**12**) : **A, PN; HT**
2-Methyl-3-oxotetradecanoic acid-Pro-Aib-Aib-Aib-Aib-Ala-Ala-Ala-Aib-β-Ala-Leu-Aib-βAla-Ala-Aib-β-Ala-Aib-Aib-Aib-Ala-Arginal
Ampullosporin B (**13**): **PS; HS**
Ac-Trp-Ala-Aib-Aib-Leu-Aib-Gln-Ala-Aib-Aib-Gln-Leu-Aib-Gln-Leuol
Ampullosporin D (**14**): **PS; HS**
Ac-Trp-Ala-Aib-Aib-Leu-Aib-Gln-Aib-Aib-Ala-Gln-Leu-Aib-Gln-Leuol
Desacetyltryptophanyl-ampullosporin A (**15**) **PN; HN**
Ala^{1}-Aib^{2}-Aib^{3}-Leu^{4}-Aib^{5}-Gln^{6}-Aib^{7}-Aib^{8}-Aib^{9}-Gln^{10}-Leu^{11}-Aib^{12}-Gln^{13}-Leu^{14}-ol
Hexamethylated ampullosporin A (**16**) **PN, HN**
Ac-Trp^{1}-Ala^{2}-Aib^{3}-Aib^{4}-Leu^{5}-Aib^{6}-$Gln^{7}(Me)_2$-Aib^{8}-Aib^{9}-Aib^{10}-$Gln^{11}(Me)_2$-Leu^{12}-Aib^{13}-Gln^{14}(Me)H-Leu^{15}-OMe

Figure 1 Amino acid sequences of peptaibols used in this study and their effects on morphogenesis of *Phoma destructiva* (**P**) and induction of hypothermia in mice (**H**). The horizontal line marks the average level of conductivity in the control experiments without the presence of peptaibols. **S**: stimulation positive response: **N**: no effect; **T**: toxic in mice; **A**: antimicrobial activity against *Phoma destructiva*. HyPro: Hydroxyproline.

96 h after a single intraperitoneal administration of 10–20 mg peptaibol/kg body weight (bw). In addition, the locomotor behaviour of the animals was observed and estimated.

RESULTS AND DISCUSSIONS

Figure 1 shows the biological activities and amino acid sequences of the peptaibols and lipopeptaibols used for comprehensive measurements of membrane activities. The biological activities are abbreviated as follows: '**P**' is the induction of morphogenesis and pigment formation in surface cultures of the fungus *Phoma destructiva*. This assay, as described earlier [3], enabled the qualitative comparison of the pigment-inducing capacity of the compounds. '**H**' is the induction of hypothermia and reduction of spontaneous locomotor activity in mice after a single intraperitoneal administration of peptaibols at a dose of 10–20 mg/kg bw. The abbreviation '**S**' signifies stimulation (positive response) of the fungal pigment formation and induction of hypothermia in mice, respectively, and '**N**' means 'no effect'. '**T**' indicates toxicity in mice at the tested dose, and '**A**' indicates the antibiotic effect of the pertinent peptaibol against *Phoma destructiva*. Figure 2 displays the membrane conductances for potassium ions as measured in the presence of a 0.5 μg/ml peptaibol concentration at both sides of the BLM

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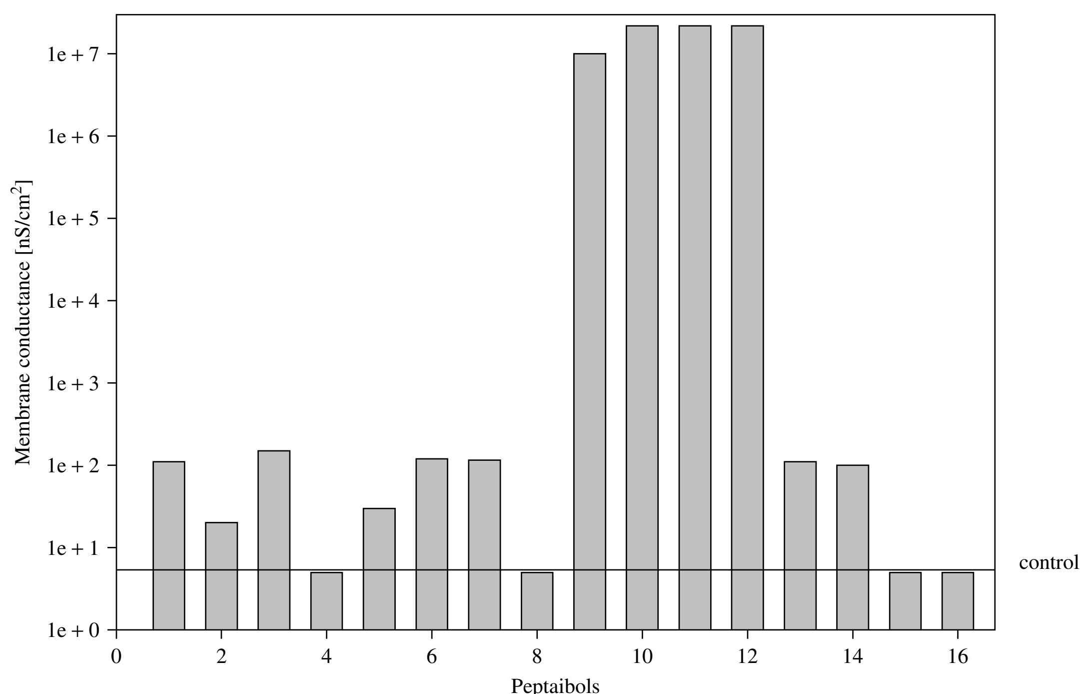

Figure 2 Increase in membrane conductance in presence of 0.5 μg/ml peptaibols (**9** and **10**: 0.25 μg/ml). For numbering see Figure 1. Control: zero current measured in the absence of peptaibols.

in the measuring cell. Alamethicin F-30 (**9**) and chrysospermin C (**10**) were used at a lower concentration (0.25 μg/ml) because of their high activity. The zero level of membrane conductance without the addition of peptaibols (control) amounted to 5 nS/cm^2 ±1 nS/cm^2.

Very high membrane activities were found with 19–20mer peptaibols such as alamethicin F-30 (**9**), paracelsin A (**11**), chrysospermin C (**10**) and 20mer lipopeptaibol texenomycin A (**12**, Figure 2). In comparison with these compounds, the 11–17mer peptaibols such as ampullosporin A, B, D (**1**, **13**, **14**, 15mer), trichofumins A (**2**, 11mer) and C (**3**, 13mer), bergofungin B (**5**, 15mer), cephaibols B and C (**6** and **7**, 16mer) were distinguishable by a relatively moderate permeabilization of black lipid membrane for potassium ions. Bergofungin A (**4**, 15mer) and cephaibol E (**8**, 16mer) were inactive under these conditions, e.g. the conductance measured in the presence of 0.5 μg/ml was the same as in the control without these peptaibols.

At a comparable concentration, the highly membrane-active peptaibols such as alamethicin F-30 (**9**) and chrysospermin C (**10**) inhibited the growth of the fungus *Phoma destructiva* in the surface culture as shown by the appearance of an inhibition zone around the agar well. However, if the peptaibol concentration given to the agar well was reduced by a factor of 20 a weak stimulation of brownish melanin-type pigment formation was observed. Paracelsin A (**11**) and texenomycin A (**12**) inhibited fungal growth, too, but did not stimulate pigment formation even at a lower concentration (Figure 1). At a dosage of 20 mg/kg bw all these highly membrane active peptaibols and lipopeptaibols were toxic to mice. Long acting hypothermia as an indicator of neuroleptic activity [3] was not observed due to the short life-span of the animals after drug administration. Otherwise the moderately active peptaibols that increased membrane conductance up to 400 nS/cm^2, such as ampullosporin A, B, D (**1**, **13**, **14**), trichofumins A (**2**) and B (**3**), cephaibols B (**6**) and C (**7**) were active both as inducers of pigment formation by *Phoma destructiva* and hypothermia in mice. Moreover, they neither displayed antibiotic effects towards the fungus *Phoma destructiva* nor acute toxicity. Bergofungins A (**4**) and cephaibols E (**8**) showing low or even no membrane activity were inactive under the same conditions. Bergofungin B (**5**) displaying moderate membrane activity afforded fungal morphogenesis at a 20-fold higher concentration than ampullosporin A but was inactive in mice at a dose of 20 mg/kg.

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Ampullosporin A (**1**) and bergofungins (**4**, **5**) are 15mer peptaibols differing in amino acid sequence. The observed differences in the membrane activities and biological effects of these 15mer peptaibols suggested that the constituting amino acids and physico-chemical properties of peptaibols are essential factors in determining the permeabilization of bilayer membranes. This conclusion was supported by investigation of four ampullosporin-type structures that were obtained either as biosynthetic homologues of ampullosporin A (**1**) from cultures of *Sepedonium ampullosporum* (ampullosporins B (**13**) and D (**14**)) [4] or by semisynthetic modification of ampullosporin A (**15** and **16**). Ampullosporins B and D (**13**, **14**) increased the membrane conductance in a manner comparable to ampullosporin A and showed the same biological effects on *Phoma destructiva* and mice at the same concentration [14,15]. However, desacetyltryptophanylampullosporin A (**15**), and ampullosporin A which was hexamethylated at the δ-carboxylamide groups of glutamine residues and the hydroxyl group of leucinol (**16**) neither increased membrane conductance nor showed any activity as inducers of fungal morphogenesis and hypothermia in mice.

The results shown in Figure 2 suggest a correlation of the biological activities of peptaibols, such as induction of pigment formation by *Phoma destructiva* and hypothermia in mice, to the permeabilization of the black lipid membrane to potassium ions. However, as a prerequisite for the above biological activities, the membrane activity should be moderate. As was shown with alamethicin F-30 (**9**), chrysospermin C (**10**), paracelsin A (**11**) and texenomycin (**12**), a high membrane activity was correlated with antimicrobial activity against *Phoma destructiva* and high toxicity in mice. Thereby the strong membrane permeabilization by these peptaibols could damage the cells of microbes and animals, and thus neither induction of fungal morphogenesis nor hypothermia in mice were observed. Otherwise no or low membrane activity under these condition appeared to be a characteristic of the structures showing no biological activity at a comparable concentration and dosage, respectively.

The results suggest that there are major differences in the relative membrane activities of peptaibols depending on the length of the peptide chain and the constituting amino acids. In particular, short-chain peptaibols with <7 amino acids displayed moderate membrane activities compared with 19 to 20mer representatives. Apparently, their molecular structure supports the formation of comparably unstable membrane pores and thus the leakage of cells should be moderate. This activity could explain the stimulation of pigment formation of *Phoma destructiva* by some peptaibols. Thus it is known that the morphogenesis and secondary metabolism of differentiating microbes is induced by nutrient starvation and by a down-shift of cellular energy metabolism [16]. At present it is not clear whether the induction of hypothermia in mice by some peptaibols is related to pore formation and membrane permeabilization. However, the results support the view that moderate membrane activity is a prerequisite of induction of long acting hypothermia and depression of locomotor activity in mice. To show this effect any active drug has to enter the central nervous system (CNS) and to interfere with neuronal receptors and signal transduction pathways. Almost all of the neuroactive, low-molecular weight drugs so far known are lipophilic and amphiphilic structures. In this respect smaller peptaibols display comparable properties. However, to elucidate the effect of peptaibols on neuronal function further investigations will be necessary.

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