Lipohexin, a New Inhibitor of Prolyl Endopeptidase from *Moeszia lindtneri* (HKI-0054) and *Paecilomyces* sp. (HKI-0055; HKI-0096)

I. Screening, Isolation and Structure Elucidation

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Lipohexin was isolated as a novel lipohexapeptide (**I**) ($C_{39}H_{68}N_6O_9$) from three fungal strains, *Moeszia lindtneri* HKI-0054, *Paecilomyces* sp. HKI-0055 and *Paecilomyces* sp. HKI-0096. The structure was elucidated by detailed mass spectrometric and NMR experiments. The proline-containing peptide displays moderate antibacterial activity against *Bacillus subtilis* ATCC 6633 and inhibits competitively the prolyl endopeptidase from human placenta.

Prolyl endopeptidase has been suggested to play an important role in the biological regulation of peptide hormones such as vasopressin, oxytocin, substance P, angiotensins and others[1]. Moreover, alterations of enzyme level and activity seem to be associated with several human disorders, such as ALZHEIMER's disease, thrombosis, AIDS and cancer[1]. Recently, new inhibitors such as poststatin[1~3] and eurystatins[4,5] were discovered in microbial cultures. Since prolyl endopeptidase cleaves preferably the amide bond between the carboxyl residue of an endogenous L-proline and a neighboured amino acid it could be suggested that other naturally occurring peptide-type inhibitors could also contain a proline moiety. In the prescreening for new bioactive metabolites we used electrospray mass spectrometry (ESI-MS, CID-MS/MS)[6,7] as a powerful tool for the detection of new peptides in crude culture extracts of microorganisms[8~10]. Our previous results suggested that linear peptides, such as peptaibols and lipopeptides are particularly well detectable by this technique, even when occurring in low amounts in presence of a high level of impurities[8,10]. In same cases, collision-induced fragmentation of single ions (*e.g.* CID-MS/MS of "quasi" molecular ions) supplied conclusive information about the occurrence of amino acids within a peptide chain due to the diagnostic m/z differences of $[M-H_2O]^+$ between two fragment ions[8~10]. Otherwise, purified samples could be subjected to special MS investigations (*e.g.* cone-voltage fragmentation (CVF-MS/MS)) to uncover the presence of proline residues[6,7].

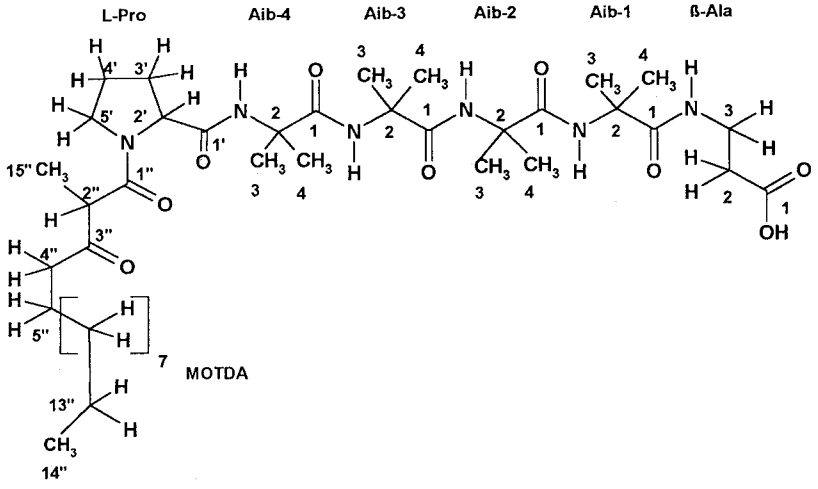
Here we report the discovery, isolation and structural elucidation of lipohexin (**I**; Fig. 1) as a new lipohexapeptide occurring in the mycelium of three fungal strains, *Moeszia lindtneri* (HKI-0054) and *Paecilomyces* sp. (HKI-0055; HKI-0096). In a second paper[11] the inhibition of prolyl endopeptidase from human placenta by lipohexin (**I**) will be described in detail.

Screening of Fungal Extracts for Peptide Metabolites

Ethyl acetate extracts of each 50 ml of microbial cultures were prepared and evaporated to dryness. The extracts were dissolved in methanol, highly diluted and injected directly in the electrospray ion source of a triple quadrupole mass spectrometer. Suggested $[M+H]^+$ ions with $m/z > 500$ were investigated by collision-induced dissociation (CID-MS/MS; argon as a target gas) to yield daughter ions representing characteristic amino acid constitutents of hydrophobic peptides such as *e.g.* α-aminoisobutyric acid (Aib); $[M-H_2O]^+$: m/z 85). By this way three fungal strains, *Moeszia lindtneri* (HKI-0054) and *Paecilomyces* sp. (HKI-0055 and HKI-0096), were found as producers of a new metabolite (**I**; Fig. 1a) with m/z 765 $[M+H]^+$, which contained Aib according to CID-MS/MS and FAB-MS experiments. As in the case of the recently discovered helioferins[8] and chrysospermins[10], the proline moiety of **I** was hardly detectable by simple FAB-MS and ESI-CID-MS/MS experiments.

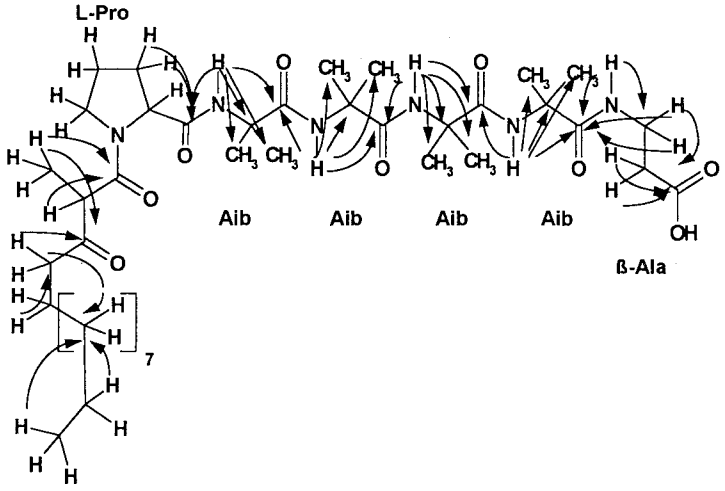
Fig. 1.

a) Structure of lipohexin (**I**).



MOTDA: 2-methyl-3-oxo-tetradecanoic acid.

b) Selected heteronuclear long-range couplings as detected in the HMBC spectrum of **I**.



Hence, cone-voltage fragmentations in the ion source and elucidation of the grand-daughter ion spectra was a suitable mass-spectrometric technique to solve this problem.

Occurrence, Production and Isolation of Lipohexin

Moeszia lindtneri (HKI-0054) and *Paecilomyces* sp. (HKI-0055, HKI-0096) from the strain collection of the Hans-Knöll-Institute of Natural Product Research, Jena (Germany), were cultivated as surface cultures at 25°C in 500-ml Erlenmeyer bottles containing 100 ml medium composed as follows (g/liter): glycerol (30), glucose (10), peptone (5), NaCl (2), zeolite (0, 1), agar (1) dissolved in distilled water (1 liter). The bottles were sterilized at 121°C for 20 minutes. Each surface culture was inoculated with a 2 cm^2 area of a 21 days agar culture. After 14 days of cultivation, 40 liters of the cultures of both strains were filtrated. The mycelium cake was extracted twice with 5 liters of methanol, the methanol was removed *in vacuo* and the aqueous residue was reextracted twice by 1 liter ethyl acetate. The culture medium broth was extracted by 20 liters ethyl acetate and the combined extracts were evaporated to dryness (yield 4.5~6.5 g). The residue was dissolved in 25 ml methanol and the solution subjected to a column (50 × 10 cm) filled with Sephadex LH-20 in methanol. At first, fractions were eluted with methanol containing lipohexin (**I**), as detected by electrospray ionization MS. The fractions containing $[M+H]^+$ *m/z* 765 and $[M+Na]^+$ *m/z* 787 were pooled

and purified by preparative isocratic HPLC (Spherisorb RP_{18}; 25 mm × 250 mm, CH_3CN-H_2O 83:17, (v/v), 10 ml/minute, detection at 210 nm; retention time 11.2 minutes, yield 120~150 mg). In addition to lipohexin (**I**) the methyl ester of **I** peaked in preparative HPLC fractions of *Paecilomyces* sp. HKI-0055 subsequent to **I** (retention time 12.5 minutes, yield 10 mg).

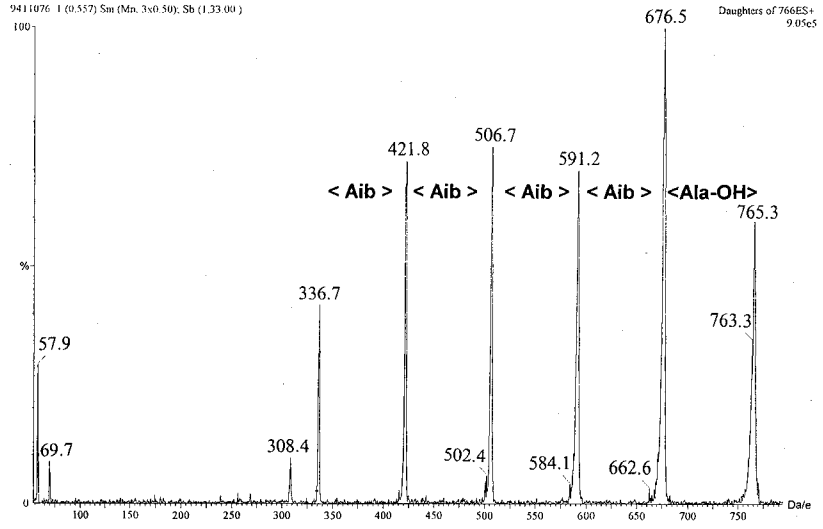
Physical Properties and Structure of Lipohexin (**I**; Fig. 1a)

Lipohexin (**I**) (white crystals) from all strains melted at 191°C with decomposition. It dissolves well in chloroform and methanol but is insoluble in water. The molecular formula $C_{39}H_{68}N_6O_9$ and mass of **I**: ($[M+H]^+$: *m/z* 765.5048; calcd. 765.5051) were determined by high-resolution (HR) FAB mass spectrometry using 3-nitrobenzyl alcohol as matrix[8,10]. The amino acid composition of I (β-alanine, α-aminoisobutyric acid) was determined initially from the FAB fragmentation behaviour[12]. In addition to the diagnostic fragments of the above amino acids[12], a fragment with *m/z* 336 could be ascribed to the prolyl-2-methyl-3-oxotetradecanoic acid moiety (Pro-MOTDA).

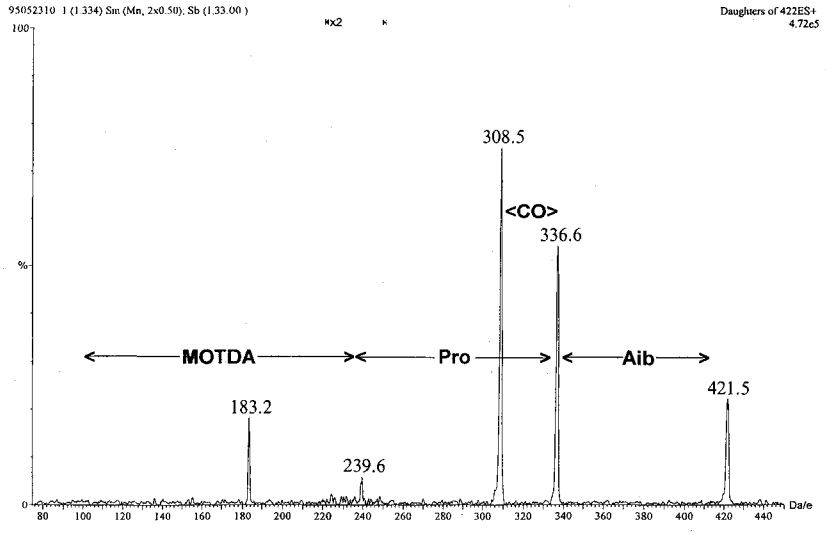
This suggestion was confirmed by the electrospray ionization CID-MS/MS experiments showing the presence of four Aib units and one β-alanine (Fig. 2a).

Fig. 2. Electrospray mass spectra of lipohexin (**I**).

a) Electrospray-CID-MS/MS fragmentation pattern of the single ion peak *m/z* 765 ($[M+H]^+$).



b) ESI-MS/MS spectrum of fragment ion *m/z* 421 after cone-voltage fragmentation in the ion source.



MOTDA: 2-methyl-3-oxo-tetradecanoic acid.

MS/MS of single ion m/z 421.5 obtained after cone-voltage fragmentation of m/z 765 ($[M+H]^+$) provided the grand-daughter ion spectrum as depicted in Fig. 2b. The presence of proline is clearly recognizable as well as a terminal fragment of m/z 239 (MOTDA). The absolute configuration of the proline moiety was determined to be *S* by hydrolysis of the peptide, derivatization of the amino acids by Marfey's reagent and HPLC analysis[13]. The FT IR spectrum (in KBr) showed characteristic absorbances of λ_{max} 1424, 1626 and 1672 cm^{-1} suggesting the presence of amide structures. Conclusive evidence for the structure of **I** as shown in Fig. 1a was derived from ^1H, ^{13}C, and DEPT spectra confirming the presence of 39 carbon atoms. These could be distinguished into ten methyls, fifteen methylenes, two methines, four quarternary carbons, one oxo carbonyl, and seven peptide carbonyl carbons. Extensive 2D NMR spectroscopic investigations including COSY, TOCSY, HMQC and HMBC experiments, suggested the sequence of β-oxofatty and amino acids in the molecule (Table 1). The methyl ester of **I** obtained from *Paecilomyces* sp. HKI-0055 was distinguishable from lipohexin by m/z 779 $[M+H]^+$ in the FAB and ESI spectra, but was shown to be identical in its MS/MS fragmentation pattern.

In particular, H, C long-range couplings were helpful to clarify the methyl groups of the Aib residues and the methyl group at C2″ (52.0 ppm) of the tetradecanoic acid residue of **I** (Fig. 1b). Moreover, the overlapping multiplets of the C4″-H_A (2.69 ppm; dt) C4″-H_B (2.57 ppm; dt), C2-H_A (2.69 ppm; dt) and C2-H_B (2.63 ppm; dt) of β-Ala residue could unambiguously be assigned. The peptide structure of the novel lipohexapeptide **I** thus appears as related to that of the peptaibols[14,15], but also as distinguishable due to the inherent sequence of four α-aminobutyric acid moieties and the presence of an unreduced carboxylic terminus.

Biological Activity

Lipohexin (**I**) inhibited the activity of prolyl endopeptidase from human placenta in a competitive manner with *Ki* 3.5 μM. Kinetic details of enzyme inhibition by **I** will be described in our accompanying paper[11]. In the common agar well diffusion assay[16] **I** showed moderate antibacterial activity against Gram-positive bacteria such as *Bacillus subtilis* ATCC 6633 (17 mm diameter of inhibition zone around an agar well of 9 mm diameter in presence of 100 μg of **I**).

Table 1. ^1H and ^{13}C NMR data of lipohexin (**I**) in $CDCl_3$.

	^{13}C	^1H
β-Ala		
1	174.1 s	—
2	35.8 t	H_β 2.69 dt (16.4; 6.3)
		H_α 2.63 dt (16.4; 6.3)
3	35.6 t	H_β 3.53 (m)
		H_α 3.51 (m)
NH	—	7.88 br t (5.1)
Aib1		
1	177.2 s	—
2	57.0 s	—
3	27.2 q	1.51 s
4	24.0 q	1.58 s
NH	—	7.46 br s
Aib2		
1	174.7 s	—
2	57.0 s	—
3	26.5 q	1.47 s
4	23.5 q	1.46 s
NH	—	7.53 br s
Aib3		
1	175.6 s	—
2	56.7 s	—
3	26.2 q	1.44 s
4	23.3 q	1.42 s
NH	—	7.37 br s
Aib4		
1	174.6 s	—
2	56.8 s	—
3	26.4 q	1.43 s
4	23.6 q	1.47 s
NH	—	7.59 br s
Pro		
1′	171.8 s	—
2′	61.5 d	4.35 dd (8.0, 4.9)
3′	29.5 t	H_α 2.01 (m)
		H_β 2.30 (m)
4′	25.1 t	H_β 2.01 (m)
		H_β 2.00 (m)
5′	47.3 t	H_α 3.32 ddd (13.6, 6.6, 6.0)
		H_β 3.55 ddd (13.6, 6.8, 6.2)
MOTDA		
1″	170.3 s	—
2″	52.0 d	3.70 q (7.2)
3″	210.9 s	—
4″	40.6 t	H_α 2.57 dt (17.5, 7.5)
		H_β 2.69 dt (17.5, 6.3)
5″	29.3 t	1.61 m
6″	23.5 t	1.52 m
7″	29.1	1.26 m
8″	29.3	1.26 m
9″	29.4	1.26 m
10″	29.4	1.26 m
11″	29.5	1.26 m
12″	31.9 t	1.26 m
13″	22.7 t	1.29 m
14″	13.6 q	0.88 t (6.8)
15″	12.8 q	1.47 d (7.2)

MOTDA: 2-methyl-3-oxo-tetradecanoic acid; Pro: L-proline; Aib: α-aminoisobutyric acid; β-Ala: β-alanine.
Carbon numbering see Fig. 1a.
Chemical shifts (δ) in ppm; coupling constants in Hz; s: singlet; d: doublet; t: triplet; br: broad; m: multiplet.

Experimental

All NMR spectra were recorded in $CDCl_3$ on Bruker AC-200 and Bruker AMX 600 FT-NMR spectrometers. ^{13}C NMR spectra were measured with a 75 mM solution at 50.3 MHz. ^{1}H NMR, COSY, TOCSY and heteronuclear 2D-NMR spectra were recorded with a 21 mM solution at 600.13 MHz.

FAB mass spectra were recorded on an AMD-402 instrument of BE geometry (AMD Intectra, Harpstedt, Germany). Ions were produced by fast ion bombardment with a 12 KeV Cs^+ beam, generated in a Cs^+ gun (liquid SIMS system Intectra). Peptide solutions were mixed with 3-nitrobenzyl alcohol as matrix on the FAB probe tip as reported earlier[8,10]. Electrospray-MS and CID-MS/MS spectra were recorded on a triple quadrupole instrument Quattro (Fisons VG Micromass, Altrincham, England)[8,10].

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