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Hassallidin B—Second antifungal member of the Hassallidin family

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Abstract—The cyanobacterium *Hassallia* sp. produces a family of four compounds which exhibit a broad spectrum of antifungal activities. So far only one of these members has been isolated and its structure elucidated. In this study, we present a second member of this group. Mass spectrometry, one- and two-dimensional NMR and chiral GC-MS analysis revealed the same peptidic and fatty acid core for hassallidin B as the first member hassallidin A with an additional carbohydrate unit, a rhamnose attached to the 3-hydroxyl group of the C_{14}-acyl side chain. The antifungal potential of hassallidin B is nearly identical to that of hassallidin A.

We report on the isolation and structure determination of an additional antifungal compound belonging to the hassallidin family from the cyanobacterium *Hassallia* sp. Mass spectrometry, one- and two-dimensional NMR and chiral GC–MS analysis revealed the same peptidic and fatty acid core of hassallidin B as the known natural product hassallidin A.[1] Contrary to hassallidin A, an additional carbohydrate unit, linked to the lipid unit and identified as rhamnose, could be determined. This carbohydrate seems to be of little influence on the antifungal activity since hassallidin B showed similar MICs (minimal inhibitory concentrations) like hassallidin A, but it may be useful for modification and applicability of hassallidin B (see Fig. 1).

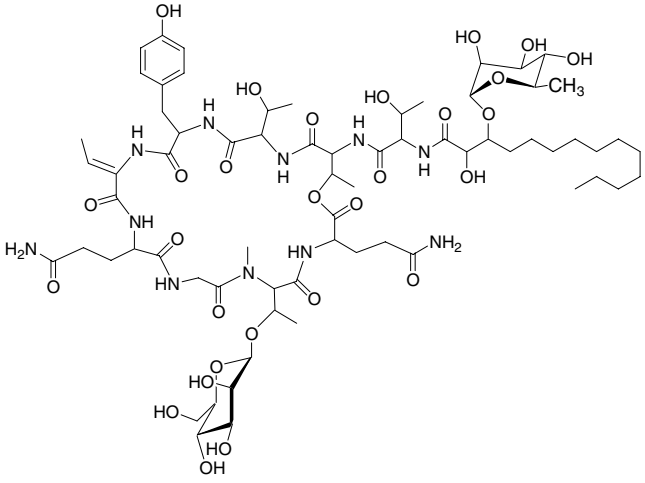

Figure 1. Hassallidin B.

Cells were grown for 30 days in modified BG-11 medium according to Welker[2] at 20 °C in 20 L flasks and were continuously illuminated and aerated. After filtration and lyophilisation, 4.3 g of dry material was obtained. The freeze-dried material was treated with MeOH to extract the active compound from the cyanobacterial biomass. After evaporation of solvent, the residue was eluted from a solid-phase extraction cartridge in preparation for an HPLC (Waters 515). The biologically active fractions, collected on eight reversed-phase HPLC runs [Waters Spherisorb S5 ODS2, 10×250 mm column; mobile phase: solvent A: H_2O/formic acid (0.05%), solvent B: acetonitrile/formic acid (0.05%); 3 mL/min; UV detection at 220 nm], were combined to yield 3.2 mg hassallidin B. The following gradient was applied: solvent B from 30% to 35% in 10 min, 35% to 70% in 30 min, 70% to 100% in 4 min and isocratic 6 min. The hassallidin B fraction eluted at 21–22 min.

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High-resolution electrospray Fourier transform ion cyclotron mass spectrometry (Finnigan ThermoQuest ESI-FTICR-MS) of hassallidin B revealed a quasi-molecular ion $[M+K]^+$ at *m/z* 1566.7085 consistent with the calculated molecular formula of $[C_{68}H_{109}N_{11}O_{28}K]^+$ (requires *m/z* 1566.70802, relative mass error $\Delta_m = 0.31$ ppm).

Amino acid analysis of the total hydrolysate was performed by chiral gas chromatography–mass spectrometry (GC–MS). Approximately 50 nM sample was hydrolysed in 200 μL of 6 N HCl (110 °C/24 h). The dry hydrolysate was derivatized to the *N*-(*O*–) trifluoroacetyl/ethyl ester and analysed by GC–MS (Agilent 6890/5973 MSD) using a 20 m × 0.25 mm Lipodex E/PS255 (30:70) capillary column.

The following amino acid composition could be determined: D-Tyr, D-Thr, L-Thr, D-allo-Thr, *N*-MeThr, D-Glu, L-Glu, Gly and dehydroaminobutyric acid (Dhb). The sugar and fatty acid analysis was also performed by GC–MS. The sample was heated at 70 °C for 16 h with 0.65 N HCl/abs. MeOH. Excess methanol was evaporated off. The dry residue was treated with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA)/acetonitrile (1:1) (60 °C/30 min). The derivatized carbohydrates and fatty acid were analysed directly by GC-MS on a DB-5 capillary (J+W, Folson).

The sugar analysis revealed the presence of rhamnose and mannose. The fatty acid was identified as dihydroxytetradecanoic acid (Dht) by comparison of its native mass with that of the corresponding TMS-methyl derivative.

Mass spectral analyses were performed on a matrix-assisted laser desorption/ionisation time-of-flight mass spectrometer (Per-Septive Biosystems Voyager-DE PRO MALDI-TOF). Sample application for MALDI-TOF measurements was carried out directly on sample plates with a mixture of 1 μL matrix (saturated 2,5-dihydroxybenzoic acid in 50% acetonitrile, 0.3% TFA) and 1 μL of a 50% MeOH solution containing about 0.2 μg hassallidin B.

The monoisotopic mass of hassallidin B was determined in positive ion reflector mode by using delayed extraction (DE). Additional structural information were obtained by the fragmentation pattern using the post-source decay (PSD) modus of monoisotopic peak $[M+H]^+$. Formation of the following fragment ion was detected and interpreted as: *m/z* 1382: M—[*m/z* 146 (rhamnose)], *m/z* 1220: M—[*m/z* 308 (mannose + rhamnose)], *m/z* 1092: M—[*m/z* 436 (mannose + rhamnose + Gln)], *m/z* 920: M—[*m/z* 608 (mannose + rhamnose + Gln + Gly + MeThr)] and m/z 792: M—[*m/z* 736 (mannose + rhamnose + Gln + Gly + MeThr + Gln)], *m/z* 546: M—[*m/z* 982 (mannose + rhamnose + Gln + Gly + MeThr + Gln + Tyr + Dhb)].

The chemical composition of hassallidin B was analysed using multidimensional NMR spectroscopy. Since hassallidin B and hassallidin A are closely related, the results of the latter could be transferred to the novel peptide. Nevertheless a full set of two-dimensional spectra as described for hassallidin A[1] was recorded (DRX600 and DMX750, Bruker Biospin) for hassallidin B and all examinations were performed with hassallidin B. The similarity of the $^1H,^{13}C$ HMQCs confirmed that the two compounds are in fact identical with the exception of the attached rhamnose moiety. The only differences expected in the HMQC were additional signals from the rhamnose and changes in the signals of the attachment site of the additional sugar moiety. Originally we assumed that rhamnose is attached to the mannose forming a disaccharide, but major changes in the resonances of the fatty acid (positions 2 and 3, data not shown) were observed. The attachment of the rhamnose to the anomeric carbon to position 3 of the fatty acid could subsequently be demonstrated in the $^1H,^{13}C$ HMBC. Fig. 2 shows a region of the HMQC and the

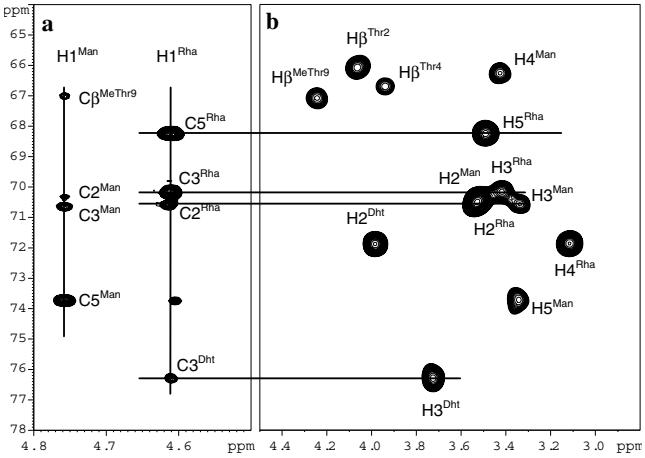

Figure 2. Two-dimensional NMR spectra of hassallidin B. (a) Region of the $^1H,^{13}C$ HMBC, the attachment of the mannose and the rhamnose to the core of the molecule are demonstrated. (b) Region of the $^1H,^{13}C$ HMQC exhibiting the resonances of the sugars, the threonine Cβs and carbon 2 and 3 of the fatty acid (Dht).

Table 1. MICs and MFCs of hassallidin B and hassallidin A against reference strains of *Candida* species and *Cryptococcus neoformans*

	Hassallidin B (μg/ml)		Hassallidin A (μg/ml)	
	MIC	MFC	MIC	MFC
C. parapsilosis ATCC 22019	8	16	8	8
C. krusei ATCC 6258	8	16	8	8
C. albicans SC 5314	8	8	4	8
C. albicans ATCC 44374	8	16	4	4
C. albicans ATCC 90028	8	8	4	4
C. albicans ATCC 24433	16	16	4	4
C. glabrata DSM 11950	16	16	8	8
C. glabrata ATCC 90030	16	16	8	8
C. parapsilosis ATCC 90018	16	>16	8	16
C. tropicalis ATCC 90874	8	16	8	8
C. tropicalis ATCC 750	16	16	4	4–8
C. krusei ATCC 90878	16	16	8	8
C. neoformans ATCC 34543	8	8	4	8
C. neoformans DSM 6973	8	8	4	8
C. neoformans CBS 6955	8	8	4	8
C. neoformans ATCC 90112	8	8	4	8

HMBC of hassallidin B. A correlation of the proton in position 1 of the rhamnose to the carbon in position 3 of the fatty acid proves the attachment unequivocally.

For the assessment of the antifungal effect of hassallidin B and its comparison with hassallidin A, minimal inhibition concentrations (MICs) and minimal fungicidal concentrations (MFCs) against selected yeast species were determined. Results are listed in Table 1. Testing was done by microdilution assay adopting the guidelines of the NCCLS document M27-A2.[3] Growth inhibition was measured by visual reading and a reading mirror as well as spectrophotometrically (microplate reader MR 5000, Dynatech laboratories). Scoring was done according the NCCLS.[3]

The minimal fungicidal concentrations (MFCs) were performed by determining the number of CFUs (colony forming units) of the optically clear wells after the end of the incubation. CFU counting was done by plating an aliquot of the wells onto Sabouraud's dextrose agar and incubation at 35 °C for 48 h. The MFC is defined as the lowest concentration killing at least 99% of the inoculum.

It could be shown that the MICs of hassallidin B were similar to those of hassallidin A, both showing fungicidal activity. The presented data on the antifungal activity of hassallidin B against selected yeasts suggest that this substance does not result in a different mode of action compared to hassallidin A. The additional carbohydrate moiety does not play a decisive role in the antifungal mode of action. However, due to the additional hydrophilic unit, hassallidin B shows a better water solubility. Within the drug design feature the biosynthetic modification could be important for an improved bioavailability by enhanced water solubility, keeping the broad spectrum of antifungal activity.[4] So, hassallidin B can be regarded as an excellent source for the development of new antifungal drugs.

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