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Glomosporin, a Novel Antifungal Cyclic Depsipeptide from *Glomospora* sp.

I. Production, Isolation, Physico-chemical Properties and Biological Activities

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A novel antifungal cyclic depsipeptide termed glomosporin, which has a fatty acyl side chain, was isolated from a barley solid culture of *Glomospora* sp. The strain was isolated from fallen pine leaves collected in Fukusima Prefecture, Japan and identified as *Glomospora* sp. BAUA 2825.

Glomosporin was purified by butanol extraction followed by preparative HPLC. Glomosporin showed antimicrobial activity against fungi including clinically important *Aspergillus fumigatus*.

The incidence of fungal infection is still increasing. Amphotericin B, which was discovered several decades ago, has many severe side-effects, but is still used as one of the main therapeutical agents for fungal infections, especially thouse caused by *Aspergillus fumigatus*. It is clear that we need a new antifungal agent with a low toxicity, active against the clinically important fungus, *Aspergillus fumigatus*.

During the course of a screening program for novel antifungal agents from secondary metabolites of fungi grown on a solid barley medium, we discovered a novel compound named glomosporin (Fig. 1) from a culture of *Glomospora* sp. BAUA 2825.

In this communication, we report the production, isolation, physico-chemical properties and biological activities of glomosporin. The producing strain is *Glomospora* sp.[1] which is the second instance of isolated genus reported so far. Detailed studies on taxonomy of the strain will be published separately[2]. Structure elucidation of the compound is described in an accompanying paper[3].

Materials and Methods

High Performance Liquid Chromatography (HPLC)

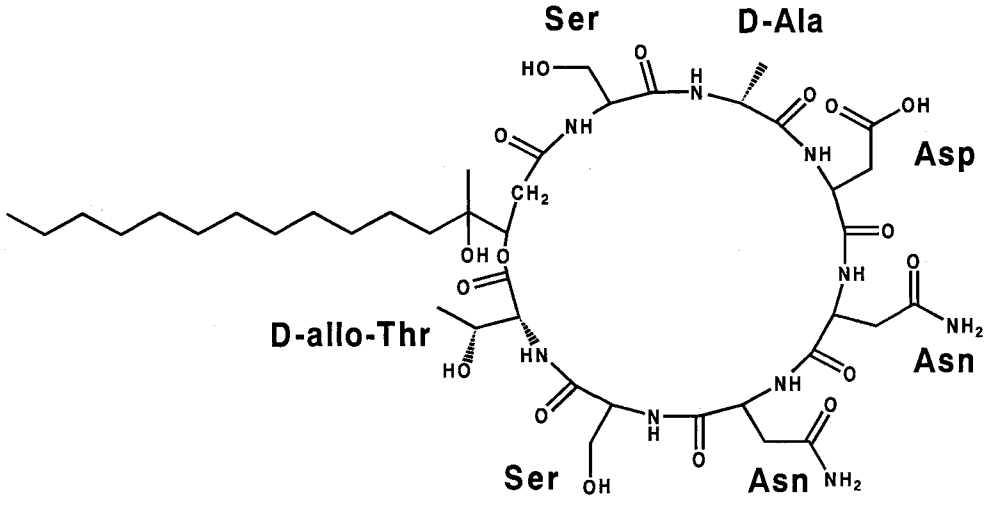
Analytical HPLC was carried out using a Shimazu LC-10AD system equipped with a diode array detector (SPD-M10A, Shimazu) and Capcell Pak C18 UG120 4.6×100 mm (Shiseido) with a CH_3CN gradient in 0.05% TFA; a linear gradient from 15% CH_3CN in 0.05% TFA to 85% CH_3CN (0~15 minutes), 85% CH_3CN (15~20 minutes), 100% CH_3CN (20~25 minutes) and 15% CH_3CN (25~32 minutes); flow rate, 1.0 ml/minute. Under these conditions, glomosporin was eluted at a retention time of 11.8 minutes.

Preparative HPLC was carried out on the same system using Capcell Pak C18 UG120 20×250 mm (Shiseido); mobile phase, H_2O - CH_3CN (60 : 40) in 0.05% TFA and a flow rate of 9.9 ml/minute.

Antimicrobial Activity

The antimicrobial activity of glomosporin was determined by a broth microdilution method using RPMI1640 medium buffered with MOPS (3-(*N*-

Fig. 1. Structure of glomosporin.



morpholino)propanesulfonic acid) in accordance with the National Committee for Clinical Laboratory Standards (NCCLS) document M27-A[4] with a slight modification in the criteria for reading of MICs against *Aspergillus* species. The minimum inhibitory concentrations (MIC) was observed after 24 hours incubation at 35°C for *Aspergillus*, 48 hours for *Candida* and 72 hours for *Cryptococcus*. The lowest concentration with turbidity under 20% of growth control was read as MIC for *Candida* and *Cryptococcus*. For *Aspergillus* species, the lowest concentration with growth reduction rate the "score 1" by Espnel-Ingroff[5] (75% reduction relative to growth control) or below was read as the MIC.

The antimicrobial spectra were determined using paper discs (i.d. 8 mm, ADVANTEC, 50 μl of 1 mg/ml solution) containing 50 μg glomosporin. *Aspergillus fumigatus* tested was grown on Sabouraud agar medium, other fungi on PDA (potato dextrose agar), yeasts on YMA (yeast malt extract agar) and bacteria on LB (Luria-Bertani broth) agar medium. The inhibitory zone was observed after 48 hours incubation at 35°C for *A. fumigatus*, 48 hours incubation at 25°C for other fungi and yeasts and 24 hours incubation at 37°C for bacteria.

Fermentation

A slant culture of fungal strain BAUA2825 was inoculated into 5 test tubes (25 mm i.d.) containing 10 ml of a sterile culture medium consisting of soluble starch 2.0%, glucose 1%, soybean flour 1.5%, malt extract 0.5%, $MgSO_4$ 0.05%, KH_2PO_4 0.05%, V8 vegetable juice 10% (V/V), and potato dextrose 10% (V/V). The test tubes were shaken on a reciprocal shaker (250 rpm) at 25°C for 72 hours.

A half ml of this seed culture was transferred into each of 100 test tubes (30 mm i.d.) each containing 10 g of pressed barley medium supplemented with 0.5% malt extract. The inoculated test tubes were incubated for 10 days at 25°C. Fermentation profile was monitored daily by HPLC. The solid cultured mass was extracted with MeOH, which was filtered and subjected to analytical HPLC.

Fig. 2. Scanning electron micrograph of *Glomospora* BAUA2825.

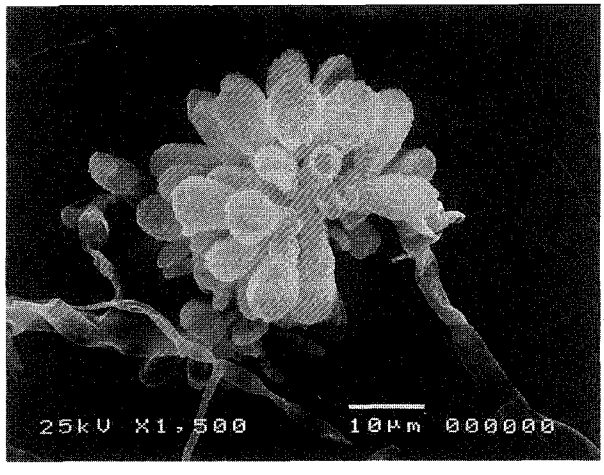
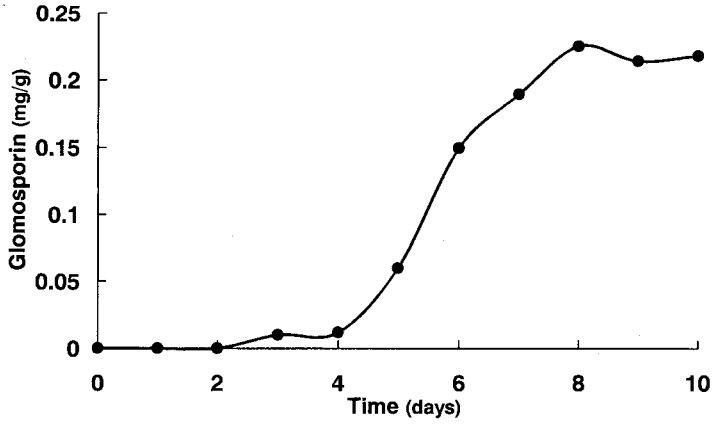


Fig. 3. Time course production of glomosporin.



Results

Producing Organism

Fungal strain BAUA2825 was isolated from a fallen pine leaves collected at Higasishirakawa, Fukusima, Japan. From the taxonomic studies[2] including cultural, physiological and morphological characteristics, the strain was found to belong to the genus *Glomospora* (Fig. 2). This strain has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan under the accession number FERM P-17066.

The producing strain is the second instance of *Glomospora* sp. isolated so far and the detailed taxonomic studies of the strain will be published separately[2].

Fermentation

The production of glomosporin was carried out by solid state fermentation of *Glomospora* sp.

A typical time course of the glomosporin production is shown in Fig. 3. The production of glomosporin was measured by analytical HPLC as described above. The production of glomosporin started after 3 days and reached the maximum after 8 days cultivation.

For routine screening of fungal metabolites, three fermentation methods have been adopted. These are; liquid medium with agitation, liquid medium without agitation and solid substrate without agitation (stationary fermentation). Glomosporin was detected only with the stationary fermentation method. To confirm that glomosporin is produced only by stationary fermentation, the producing strain was cultured in liquid medium supplemented with ground barley (used as a matrix), with agitation and glomosporin production was estimated by HPLC analysis. Glomosporin was not detected (data not shown).

Fig. 4. Isolation and purification procedure of glomosporin.

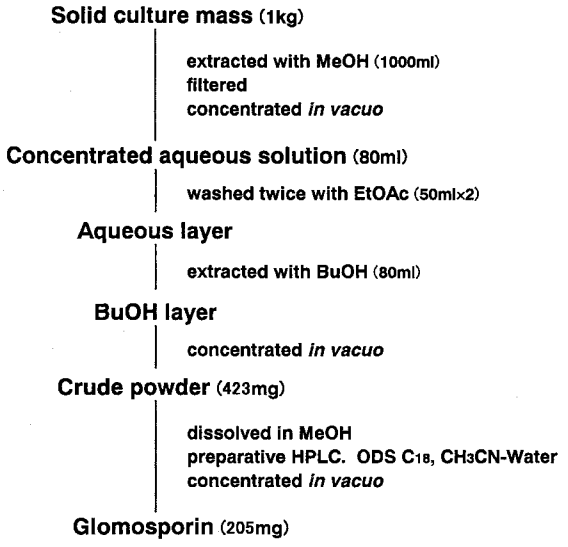
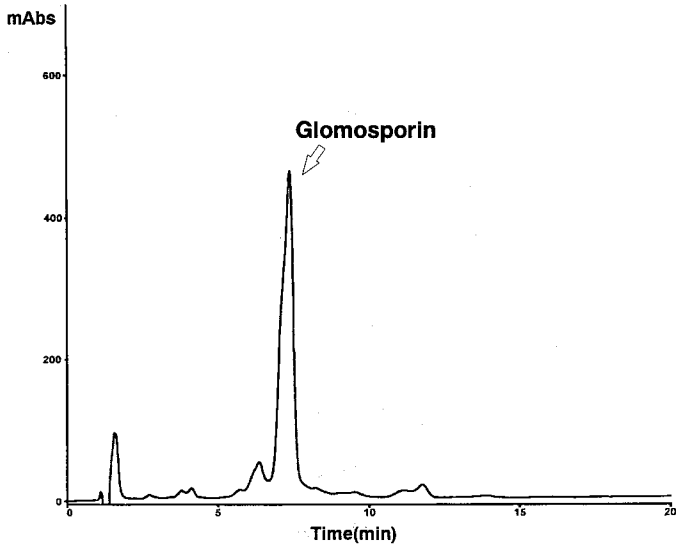


Fig. 5. Preparative HPLC of glomosporin.



Isolation and Physico-chemical Properties

A flow diagram of the isolation procedure of glomosporin is shown in Fig. 4.

The solid culture mass (1 kg) was extracted with 1 liter of MeOH. The extract was filtered and concentrated under reduced pressure at 40°C to a small volume (50 ml). The concentrated aqueous solution (80 ml) was washed twice with ethyl acetate (80 ml). The aqueous layer was extracted with BuOH (80 ml) and the BuOH layer was evaporated to dryness under a reduced pressure at room temperature. The dried material (423 mg) was dissolved in a small amount of MeOH and subjected to preparative HPLC. The preparative HPLC was carried out under the conditions as described above. The preparative HPLC profile is shown in Fig. 5. The active fractions were combined and concentrated *in vacuo* to give 205 mg of glomosporin as a white powder.

The Physico-chemical properties of glomosporin are summarized in Table 1. The molecular formula of glomosporin was determined to be $C_{42}H_{71}N_9O_{17}$ by HRFAB-MS ($[M+H]^+$ *m/z* 974.5026, calcd, 974.5046). The amino acid composition of glomosporin was shown to be, 1×Ala, 1×Thr, 2×Ser, and 3×Asx.

Table 1. Physico-chemical properties of glomosporin.

Appearance	**White powder**
Molecular formula	**$C_{42}H_{71}O_{17}N_9$**
FAB-MS m/z	**974[M+H]$^+$**
HRFABMS	
Found :	**974.5024 [M+H]$^+$**
Calcd :	**974.5046 [M+H]$^+$ for $C_{42}H_{71}O_{17}N_9$**
UV nm in EtOH	**End absorption**
$[\alpha]_D$	**-10.3 (c=0.09, EtOH)**

Biological Properties

As shown in Table 2, glomosporin was active against most fungi including clinically important *Aspergillus fumigatus* and yeasts, but not against *Bacillus subtillis* or *Pythium myriotylum*.

The minimal inhibitory concentrations (MIC) of glomosporin are shown in Table 3. Glomosporin showed a moderate activity (MIC, 16 μg/ml) against some strains of *Aspergillus fumigatus* and some yeasts.

The acute toxicity (LD_{50}) of glomosporin was estimated to be 25 mg/kg by intravenous injection (male CD-1 mice).

Discussion

This paper is the first report of a bioactive metabolite from the genus *Glomospora* sp. It should be mentioned that

Table 2. Antimicrobial activity of glomosporin.

Strain	Inhibition zone (mm)
Pythium myriotylum KPMS	0
Rhizoctonia solani AG-1 CsGi	23
Fusarium solani f. sp *phaseoli*	16
Trichoderma viride IFO 30498	24
Rhizopus chinensis IFO 30499	20
Cylindrocladium crotalariae 180RA	15
Aspergillus fumigatus KA-23	28
Trichophyton mentagrophytes KD-4	16
Candida albicans TIMM 3169	13
Cryptococcus neoformans KC-201	12
Aspergillus oryzae AOK12-2	20
Bacillus subtilis	0

Paper disc contained 50 μg glomosporin dissolved in 50 μl DMSO.

Table 3. Antifungal activity of glomosporin.

Strain		MIC (μg/ml)
Aspergillus fumigatus	TIMM 0069	16
A. fumigatus	TIMM 0070	16
A. fumigatus	MF-13	16
A. flavus	ATCC 9643	32
A. flavus	TIMM 0057	32
A. niger	TIMM 2814	16
A. terreus	NUD 3265	16
Candida albicans	ATCC 10259	32
C. glabrata	ATCC 90030	64
C. parapsilosis	ATCC 90018	64
Cryptococcus neoformans	ATCC 90112	32

MICs were determined by broth microdilution method using RPMI1640 medium buffered with MOPS in accordance with NCCLS document M27-A.
Incubation at 35°C for 24hr, 48hr and 72hr for *Aspergillus*, *Candida* and *Cryptococcus* respectively.

glomosporin was produced only by solid state fermentation of the *Glomospora* sp.

It has been shown that the solid state fermentation is an efficient method to find new fungal metabolites[6,7]. However, studies using the solid state fermentation for screening are rare.

Mass production using this fermentation method is difficult, however, we have been able to show that the stationary fermentation is a useful way to produce new fungal metabolites.

Acknowledgments

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