Laboratory Culture of the Myxomycetes: Formation of Fruiting Bodies of *Didymium bahiense* and Its Plasmodial Production of Makaluvamine A

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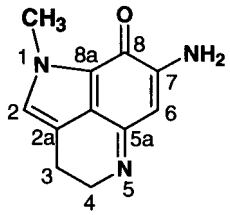
Laboratory cultures of more than 100 strains of myxomycetes were investigated, and the spore germinations of six strains were observed. The plasmodium of the myxomycete *Didymium bahiense* was cultured on oatmeal agar plates in a laboratory. The formation of fruiting bodies was observed in a plate culture. From the cultured organisms, a marine sponge metabolite, makaluvamine A (**1**), was isolated and identified on the basis of spectral data.

The myxomycetes (true slime molds) are an unusual group of primitive organisms that may be assigned to one of the lowest classes of eukaryote.[1] In the assimilative phase of their life cycle, they form a free-living, multinucleate, acellular, mobile mass of protoplasm, called a plasmodium, which feeds on living bacteria. Under certain conditions, the plasmodium undergoes sporulation to develop into small, fungus-like fruiting bodies that often have unique structures and colors. Spores, released from fruiting bodies, germinate into protozoan-like myxamoeba, which mate to form a zygote, which develops into the plasmodial stage. Although the myxomycetes have been recognized for a long time, chemical studies on the secondary metabolites of the myxomycetes are limited so far, which may be mainly attributable to a lack of adequate knowledge of their laboratory cultivation. Steglich[2] and Asakawa's group[3] have isolated several types of metabolites from field-collected samples of several myxomycete species. Cultivation of the plasmodium of myxomycetes in a considerable scale to carry out chemical studies is known only for very limited species such as *Physarum polycephalum*.[4] We recently described a preliminary study on a laboratory culture of the myxomycete *Didymium squamulosum* and its production of clionasterol.[5] Here we describe a laboratory culture of another myxomycete, *Didymium bahiense*, on a plate agar culture and its production of a marine-sponge isolate, makaluvamine A (**1**).[6] In addition, formation of the fruiting bodies of *D. bahiense* was first achieved in an artificial agar plate in this study.



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In 1998−1999, we collected 120 strains (65 species) of wild fruiting bodies of myxomycetes in Japan. Attempts on the laboratory cultures of myxomycetes were initiated by spreading spores, which were contained in the fruiting bodies, on an agar medium together with a suspension of *Escherichia coli*. After germination of the spores into myxamoebae, which were visualized by plaque formation in the *E. coli* culture, such cultures proceeded to develop into the plasmodial stage during repetitive transfer of the myxamoebic plaque to new agar plates several times. These plasmodial cultures were then able to grow by adding oatmeal to the agar medium in the absence of *E. coli*. Out of 120 strains (65 species) of wild fruiting bodies of myxomycetes, germination of spores was observed only for six strains, and development into plasmodial stage (Figure 1) was observed for five strains (Table 1). All these five strains successfully developed into spores from the plasmodial cultures in the presence or absence of light, implying that spore-to-spore cultivation, that is, rotation of one life cycle, was realized on agar plates. In addition, for only two strains, *D. squamulosum* and *D. bahiense*, was formation of fruiting bodies observed on the oatmeal agar plates. To the best of our knowledge, this is the first report of formation of fruiting bodies of *D. bahiense* (Figures 2 and 3) in an artificial agar plate, while extensive mycological studies on spore-to-spore cultivation of myxomycetes have been described on such media as cow dung, filter paper, or water agar.[7]

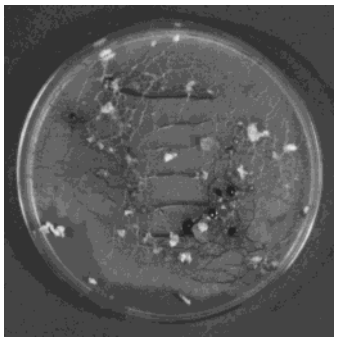

Figure 1. Plasmodium of *D. bahiense* (formed in an oatmeal agar plate culture).

The fruiting bodies of the myxomycetes *D. bahiense* (Table 1, run 4; Figure 2) were collected at Ina, Nagano Prefecture, Japan. The plasmodium of *D. bahiense* obtained as described above (Figure 1) was mass cultured in the laboratory by plate agar cultures. The harvested plasmodial cells were extracted with 90% MeOH, and the BuOH-soluble material of the extract was subjected to Si gel

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Table 1. Myxomycetes Strains That Showed Spore Germination and Plasmodial Development and Spore Formation on an Artificial Agar Plates

run	*Didymium* species	spore germination	plasmodial development	spore formation	formation of fruiting bodies
1	*D. squamulosum*[a]	observed	observed	observed	not observed
2	*D. iridis*[a]	observed	not observed	not observed	not observed
3	*D. squamulosum*[b]	observed	observed	observed	observed
4	*D. bahiense*[b]	observed	observed[c]	observed	observed[d]
5	*D. minus*[b]	observed	observed	observed	not observed
6	*D. comatum*[b]	observed	observed	observed	not observed

[a] Collected at Mt. Sanbe, Shimane Prefecture, Japan, in July, 1998. [b] Collected at Ina, Nagano Prefecture, Japan, in August, 1999. [c] See Figure 1. [d] See Figure 3.

Figure 2. Fruiting bodies of *D. bahiense* (a wild sample).

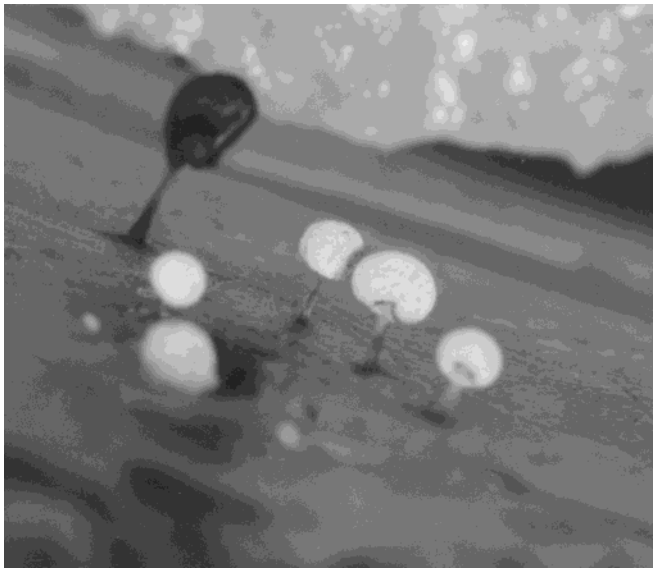

Figure 3. Fruiting bodies of *D. bahiense* (formed in a plate culture).

column chromatography, followed by separation by gel filtration on Sephadex LH-20. The fractions containing a Ninhydrin-positive spot on TLC were further purified by Si gel and Sephadex LH-20 chromatographies to give makaluvamine A (**1**), which was identified by ESIMS (*m*/*z* 202 $[M + H]^+$) and ^1H and ^{13}C NMR spectral data.

Makaluvamine A (**1**), possessing a unique pyrroloiminoquinone structure, was first isolated from a Fijian marine sponge *Zyzzya* cf. *marsailis* as a highly cytotoxic topoisomerase II inhibitor[6] and, presumably, biogenetically derived from a tryptophan. Makaluvamine A (**1**) was first isolated from myxomycetes in this study, and it seems to be noteworthy that a marine-sponge isolate was obtained from a terrestrial organism, a myxomycete, which ostensibly appears phylogenetically unrelated. These results were also reminiscent of our previous results showing that a cultured myxomycete *D. squamulosum* produced clionasterol,[5] which was previously isolated from marine sponges such as *Cliona celata* and *Hymeniacidon perleve*[8] and marine microalgae such as *Nitella flexilis* and *Chara vulgaris*. [9]

Experimental Section

General Experimental Procedures. The ^1H and ^{13}C NMR spectra were recorded on a JEOL JNM A500 spectrometer, and the 3.30-ppm resonance of residual MeOH and 49.8 ppm of CD_3OD were used as an internal references, respectively. ESIMS was obtained on a JEOL JMS-700T spectrometer. Si gel PSQ 100B (Fuji Chemical Ltd.) and Sephadex LH-20 (Pharmacia) were used for glass column chromatographies. TLC was carried out on Merck Si gel GF_{254}.

Organism and Culture. The fruit bodies of the myxomycete *D. bahiense* (order Physarales; family Didymiaceae) were collected at Ina, Nagano Prefecture, Japan, in August 1999. The spores contained in the fruit bodies were applied on an agar plate (lactose 0.1%, peptone 0.1%, K_2HPO_4 0.205%, $Na_2HPO_4 \cdot 12H_2O$ 0.083%, agar 1.5%) with a suspension of *E. coli* (0.1 mL in Nutrient media or Heart Infusion media, DIFCO). After static incubation at 25 °C in the dark for 4−5 days, myxamoebic plaque appeared, and the plaque was transferred several times to new agar plates containing the same media as above until plasmodial formation was observed. The plasmodia were then mass cultured in agar plates (the same media as above) with oatmeal (ca. 0.2 g/plate, autoclaved prior to use) for 1−2 weeks at 25 °C in the dark.

Extraction and Isolation. The harvested plasmodial cells from 121 plate cultures (9 cm ϕ) were lyophilized to give 25.2 g of material (dry wt), which was extracted with 90% MeOH (ca. 300 mL × 2). The 90% MeOH extract (1.2 g) was partitioned between EtOAc (100 mL × 3) and H_2O, and the aqueous phase was further extracted with *n*-BuOH (100 mL × 3). The *n*-BuOH-soluble material (0.7 g) was subjected to Si gel column chromatography (2.0 × 35 cm) eluted with MeOH/$CHCl_3$ (20% to 100%) and successively with $CHCl_3$/*n*-BuOH/HOAc/H_2O (1.5:6:1:1). The fraction (88 mg) eluted with 100% MeOH/$CHCl_3$ and $CHCl_3$/*n*-BuOH/HOAc/H_2O (1.5:6:1:1) was further separated by gel filtration on Sephadex LH-20 (1.8 × 50 cm; MeOH). The fractions containing a Ninhydrin-positive spot on TLC were collected and further separated by Si gel column [1.2 × 21 cm; MeOH/$CHCl_3$ (50% to 100%), $CHCl_3$/*n*-BuOH/HOAc/H_2O (1.5:6:1:1), and *n*-BuOH/HOAc/H_2O (2:1:1)] and Sephadex LH-20 (0.8 × 27 cm; MeOH) to give makaluvamine A (**1**, 8.3 mg, 0.03% yield): ^1H NMR (CD_3OD) δ 7.14 (1H, s; H-2), 5.67 (1H, s; H-6), 4.00 (3H, s; NMe), 3.86 (2H, t, $J = 7.5$ Hz; H_2-4), and 2.95 (2H, t, $J = 7.5$ Hz; H_2-3); ^{13}C NMR (CD_3OD) δ 36.4 (NMe), 131.7 (C-2), 119.7 (C-2a), 19.5 (C-3), 44.2 (C-4), 159.6 (C-5a), 88.3 (C-6), 157.7 (C-7), 169.8 (C-8), 125.0 (C-8a), and 123.9 (C-8b); ESIMS *m*/*z* 202 $[M + H]^+$; HRESIMS *m*/*z* 202.0983 (calcd for $C_{11}H_{11}N_3O$, [(M + H] 202.0980). These spectral data were consistent with those reported in the literature.[6]

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