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Zaragozic acids production from discomycetes

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In search for new zaragozic acids and their derivatives that are potent inhibitors of squalene synthase, discomycetes of the order Leotiales were cultured and their fermentation broth was assayed. Three strains in 2 unidentified species of *Mollisia* were found to produce zaragozic acid D3 and three new analogs (F-10863s). Fermentative production, productivity of F-10863s and mycological characteristics of the producers are described. Discomycetes are expected to be a biological resource providing novel bioactive compounds.

Key Words——biological resource; discomycetes; *Mollisia*; squalene synthase inhibitor; zaragozic acids.

Zaragozic acids (ZAs) are bioactive fungal metabolites known as potent inhibitors of squalene synthase (Bergstrom et al., 1993). They are a family of several compounds with a characteristic core structure (2, 8-dioxobicyclo[3,2,1]octane-4,6,7-trihydroxyl-3,4,5-tricarboxylic acid) with various side chains (Bergstrom et al., 1995). As compounds with squalene synthase inhibition activity are expected to be exploitable as hypocholesterol drugs, many researchers have endeavored to find natural compounds with such activities, and a number of ZA-related compounds have been discovered from a variety of fungi (Dawson et al., 1992; Hasumi et al., 1993; Bills et al., 1994). All of these fungi are known to belong to ascomycetes or their anamorphs. Taxonomically, they fall in three classes (Ainsworth, 1973) of ascomycetes: *Amauoascus niger* J. Schröter (Plectomycetes); *Cladosporium cladosporioides* (Fresen.) de Vries, *Curvularia lunata* (Wakker) Boedijn var. *lunata*, *Curvularia lunata* var. *aeria* (Bat., Lima & Vasconc.) M. B. Ellis, *Drechslera biseptata* (Sacc. & Roum.) M. J. Richadson & E. M. Fraser, *Exerohilum rostratum* (Drechsler) K. J. Leonard & Suggs, *Setosphaeria khatoumensis* El Shafie & J. Webster, *Sporormiella intermedia* (Auersw.) Ahmed & Cain (Loculoascomycetes and their anamorphs); *Libertella* sp. (anamorph of Pyrenomycetes). Teleomorphs of some producers are still unknown (i.e., *Leptodontium elatius* (F. Mangenot) de Hoog, *Phoma* sp., *Pseudodiplodia* sp., Sterile fungus MF 5453), but they are believed to be anamorphs of Pyrenomycetes or Loculoascomycetes, except for the last one. Most of the producing organisms belong to loculoascomycetes and their anamorphs, in particular, Pleosporales (Bills et al., 1994; Bergstrom et al., 1995). There are, however, no discomycetes or their anamorphs among the reported fungi.

Bioactive metabolites have been sought in a wide variety of fungi, in many cases soil-borne fungi. Besides soil-borne fungi, attention has been paid occasionally to plant endophytes and dung fungi (Schulz et al., 1995; Bergstrom et al., 1995). This rather biased selection of organisms to be tested may have resulted in limited variation both in structure and producing organisms of ZAs. As ZAs of related structures are obtained from various fungi, screening of underutilized fungi may result in discovery of ZAs or compounds with new structures. We therefore focused on discomycetes, in particular, on Leotiales, a group of inoperculate discomycetes with smaller apothecia, because they show a wide range of biodiversity and are relatively easily cultivated. As a result, we obtained four ZA analogs, F-10863A, B, C, D, three of which were new compounds (Tanimoto et al., 1997). This paper describes the mycological characteristics of the producing organisms and fermentative production of F-10863s. The structure and biological activity of the F-10863s has been described elsewhere (Tanimoto et al., 1997).

Materials and Methods

Small-scale fermentation and preparation of the test samples Discomycetes from a wide range of substrates were collected and isolated. Methods for collection and isolation followed Hosoya and Otani (1997). Thirty ml of the seed medium (composed of 50 g of glycerol, 5 g of malt extract (Difco), 5 g of yeast extract (Difco) and 50 g of fresh potato, 1 L water) in a 100-ml Erlenmeyer flask was inoculated with mycelia of isolates grown on potato dextrose agar medium (PDA; Nissui, Tokyo). The seed

culture was incubated for 1 wk at 23°C on a rotary shaker operating at 210 rpm. Five ml of the seed culture was transferred to inoculate another 80 ml of the same medium in a 500-ml Erlenmeyer flask, and the culture was incubated as described. Cultures were extracted with acetone and ethylacetate in acidic condition to result in extract in 10× strength concentration.

Assays Squalene synthase inhibition activity was assayed by Assay Method 2 of Tanimoto et al. (1996), which is a modification of an earlier method (Tait, 1992). For more detailed estimation of squalene synthase inhibition activity, Assay Method 1 of Tanimoto et al. (1996) was used.

Large-scale fermentation The strain SANK 10294 was used for large-scale fermentation of F-10863s. The strain was inoculated and cultured in 100 ml of medium composed of 70 g of glycerine, 30 g of glucose, 10 g of soy bean meal, 10 g of peptone, 10 g of corn steep liquor, 1 g of $MgSO_4$, 1 L water (pH adjusted to 6.5 prior to sterilization) in a 500-ml Erlenmeyer flask. The seed culture was incubated for 6 d at 23°C on a rotary shaker operating at 200 rpm. The resulting culture was transferred to inoculate the second seed at 3%, 500 ml of the same medium in a 2-L Erlenmeyer flask, which was cultivated for 2 d under the same conditions. The culture was transferred to inoculate the third seed at 3%, 30 L of the same medium in a 60-L fermentation tank, which was cultivated for 2 d at 23°C with 1.0 v.v.m. air flow. The resulting culture was transferred to inoculate 300 L of the same medium at 3% in a 600-L fermentation tank. The cultivation was carried out for 7 d at 23°C, at 1.0 v.v.m. air flow.

Isolation of F-10863s The 300 L of fermentation broth was added to an equal volume of acetone, pH was adjusted to 3 with 20% aqueous H_2SO_4, and the mixture was partitioned between 1,000 L of ethylacetate twice. The organic phases were pooled, partitioned with alkaline water, and the aqueous phase was separated. The aqueous phase was adjusted to pH 2.5 as above and re-extracted with ethylacetate. The organic phase was dried over anhydrous sodium sulfate and concentrated in vacuo. Acidified extracts was analyzed by analytical

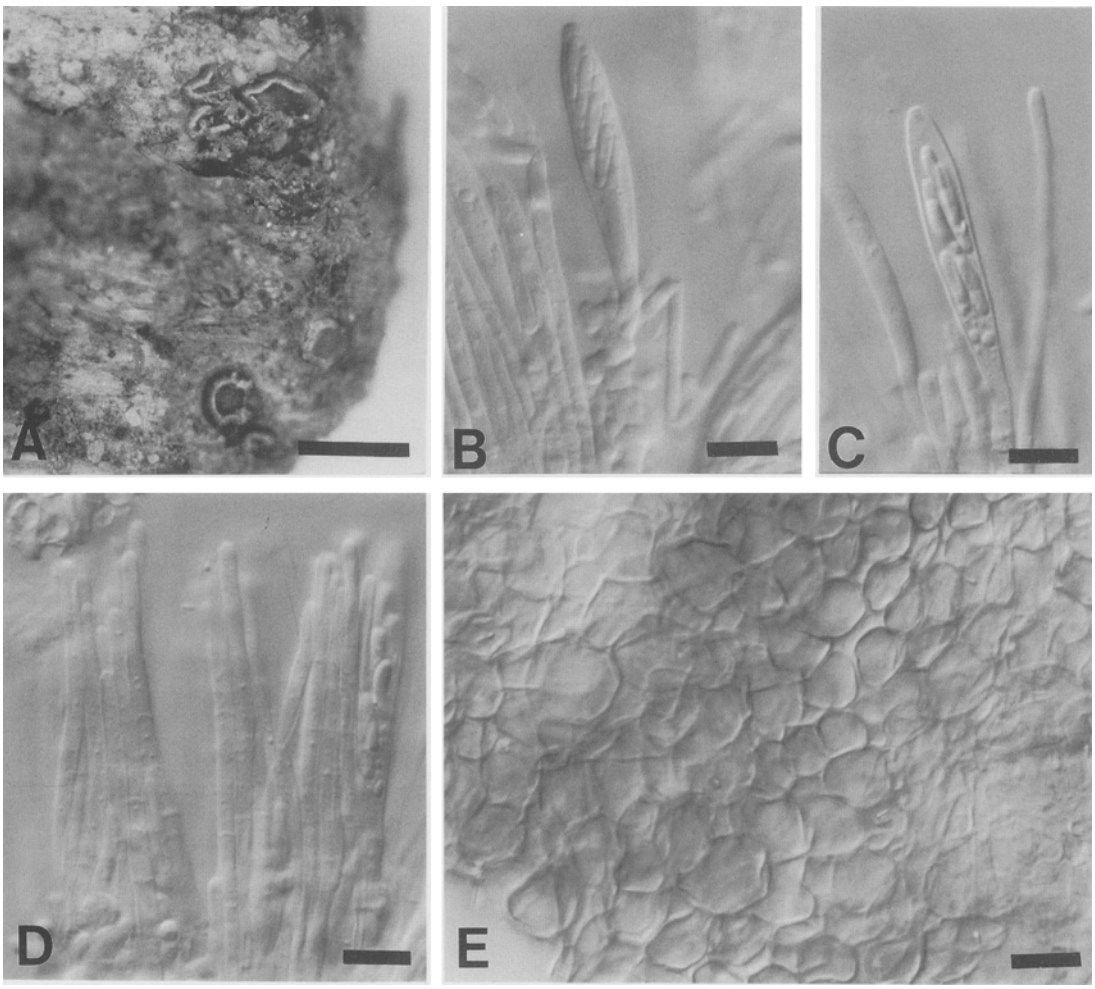

Fig. 1. *Mollisia* sp. 1 (TRL-476)
A. Dried apothecia on the substrate. B. Ascus arising from croziers. C. Ascus and ascospores. D. Paraphyses. E. Ectal cells. Scale: A, 1 mm; B-E, 10 μm.

HPLC.

HPLC separation and detection of the F-10863s The F-10863s were separated on an isocratic system on a RadialPak cartridge 8NVC184 (8 mm × 100 mm) column. The solvent was 65 : 35 (vol/vol) acetonitrile/0.3% triethylamine-phosphoric acid buffer (pH 3.2) in water, which was run at a flow rate of 1.5 ml/min at room temperature.

Observation of mycological characteristics Examination procedures for apothecial morphology followed Hosoya and Otani (1997). Micromorphological measurements were done in Melzer's reagent. To observe cultural characteristics, the strains were inoculated onto PDA and cultured for 7 d at 23°C. A one-mm agar cube containing mycelium was cut from the growing edge, and transferred to the center of another PDA plate, which was incubated at 23°C for 7 d. The colony diameter was measured, and colony morphology observed. Color indications followed Kornerup and Wanscher (1978).

Results

Assay In total, 530 isolates of discomycetes were assayed. Most of the isolates belonged to the Leotiales, in particular to the families Dermatiaceae, Hyaloscyphaceae, Leotiaceae and Sclerotiniaceae. Three isolates, namely, SANK 10294 (single-spored isolate from TRL-476 on decaying wood, Mt. Tsukuba, Tsukuba-city, Ibaraki, 24, April, 1992); SANK 19396 (multi-spored isolate from the specimen TRL-131 on decaying wood , Tomakomai, Hokkaido, 27, July, 1990); SANK 19496 (multi-spored isolate from the specimen TRL-466 on decaying wood, Kenminnomori, Saijo, Hiroshima, 5, April, 1992), were found to show squalene synthase inhibition activity in this order cited, and were left for further studies.

Fermentative production of F-10863s Four active compounds, designated here for convenience "F-10863A", "F-10863B", "F-10863C" and "F-10863D" were found in the large-scale fermentation of SANK 10294. The amounts of F-10863A, B, C, D obtained were 41.9 g

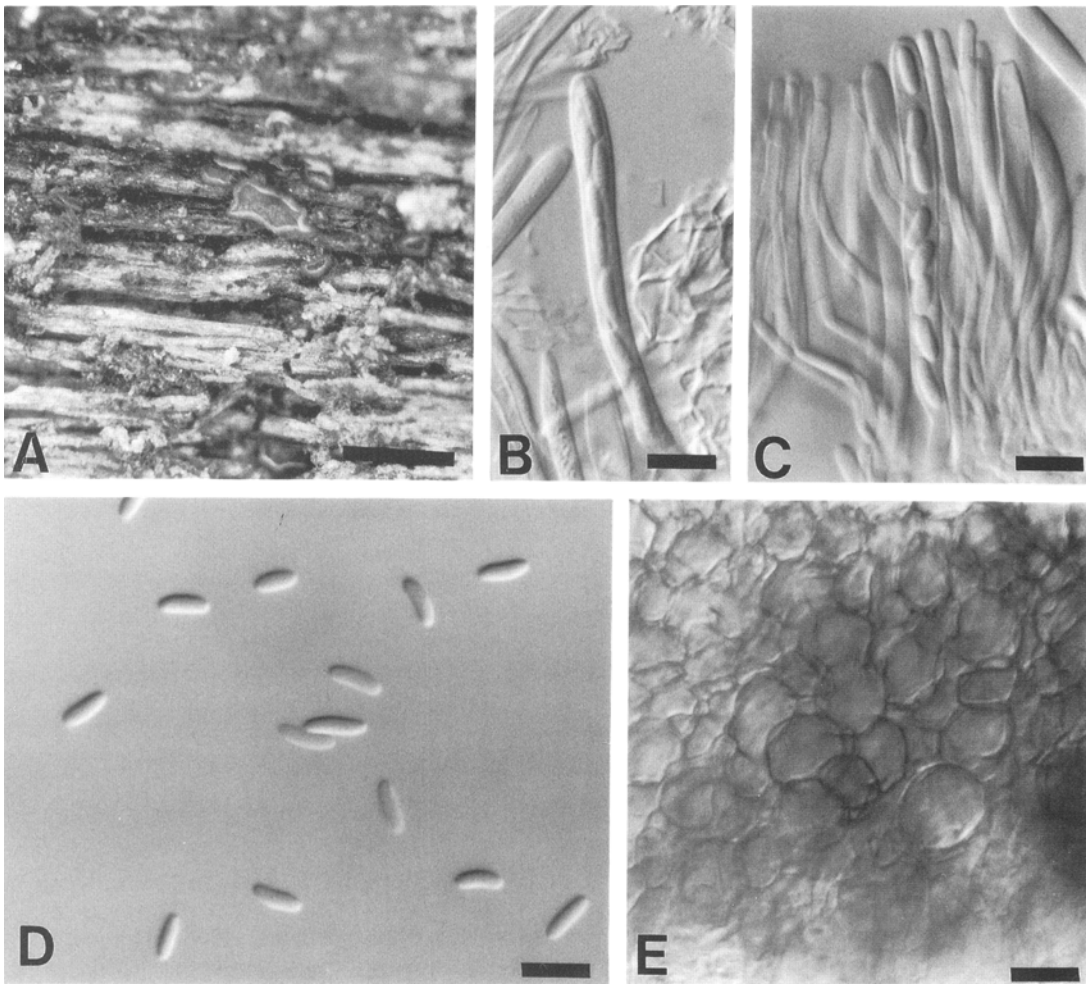

Fig. 2. *Mollisia* sp. 2 (TRL-131)
A. Dried apothecia on the substrate. B. Ascus. C. Ascus, ascospores and paraphyses. D. Ascospores. E. Ectal cells. Scale: A, 1 mm; B-E, 10 μm.

(69.8 mg/l), 14.05 g (23.4 mg/l), 0.7 g (1.17 mg/l), and 7.7 g (12.8 mg/l), respectively. The retention times were 3.3, 4.4, 4.7 and 6.8 min, respectively.

The F-10863s were characterized by a common core of 2, 8-dioxobicyclo[3,2,1]octane-4,6,7-trihydroxyl-3,4,5-tricarboxylic acid with different structures of the 6-acyl side chain and modified carboxyl group. Compound F-10863A was identical with zaragozic acid D3 in its structure (Bergstrom et al., 1995). The other three were new compounds (Tanimoto et al., 1996).

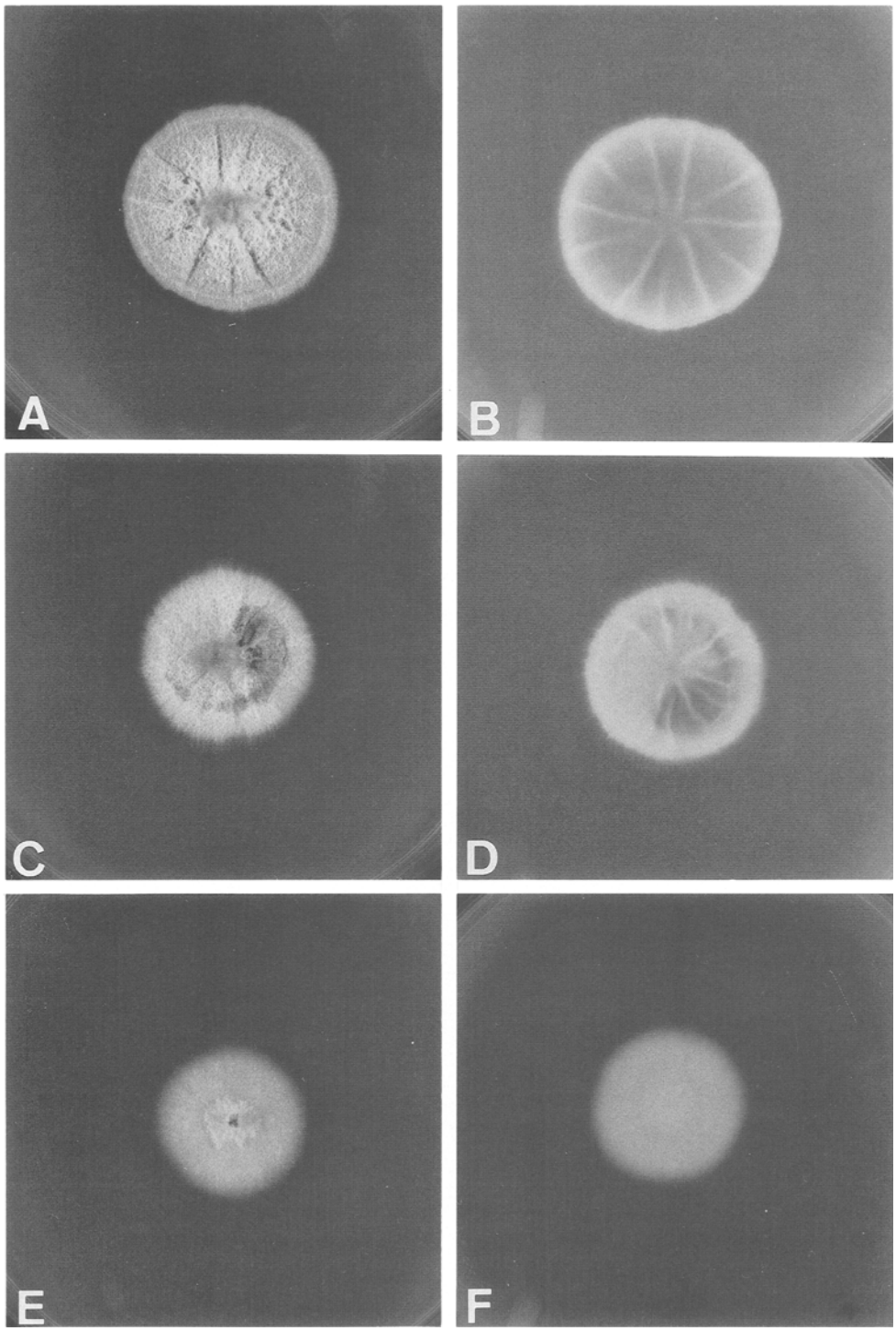

Fig. 3. Colony morphology of SANK 10294, 19494 and 19394 on PDA (23°C, 2 wk).
A. SANK 10294 (*Mollisia* sp. 1), from the surface. B. SANK 10294, from the reverse. C. SANK 19496 (*Mollisia* sp. 1), from the surface. D. SANK 19496, from the reverse. E. SANK 19396 (*Mollisia* sp. 2), from the surface. F. SANK 19396, from the reverse.

In small-scale fermentation, F-10863A and B were recognized in the culture of SANK 19396, F-10863A in SANK 19496, and F-10863A and B in SANK 10294, though they were not quantified. F-10863C and D were recognized in the large-scale fermentation of SANK 10294, but not in the small scale.

Mycological characteristics The specimens from which the strains were isolated showed similar mycological characteristics. SANK 10294 and SANK 19496 showed similar mycological properties both in culture and dried specimens from which the strains were isolated. Descriptions of the mycological characteristics follow.

TRL-476 (Figs. 1, 3A, B, 4B): Apothecia superficial, sessile, widely attached at the base, disc smooth, dull, Olive Brown (4F4), 0.4–0.8 mm in diam; receptacle concolorous to darker, nearly black; margin well defined, inrolled, concolorous with the receptacle, frilled with white minutely downy edge. Ectal excipulum of *textura globulosa*, of thin-walled dark-brown isodiametric cells, 11–18 μm in diam. Asci 50–59$\times$5.0–7.5 μm ($\bar{x}\pm$S.D.$=$ 54.3$\pm$3.0$\times$6.6$\pm$0.7 μm; n$=$20), cylindrical-clavate with a narrowed base, arising from croziers; apex conical, pore MLZ$+$ without KOH pretreatment. Ascospores 8.5–11.0$\times$2.0–2.5 μm ($\bar{x}\pm$S.D.$=$9.8$\pm$0.8$\times$ 2.3$\pm$0.2 μm; n$=$25), ellipsoid, occasionally somewhat curved, containing several small guttules when observed in MLZ. Paraphyses cylindrical, straight to slightly undulate, simple or branched near the base, septate, 3.0 μm in width, with obtuse apex.

Colony of SANK 10294 on PDA 32 mm in diam, low and dense, sulcate, umbonate at the center, Grayish Yellow (2C4), context tough and glutinous, concentrical zonation present, sector absent. Aerial hyphae funiculous, floccose at the center, forming weakly entangled strands, white, becoming Olive Gray (3E2) at the center. Margin distinct, entire, superficial. Soluble pigment not produced. Anamorph not produced.

TRL-466 (Figs. 3C, D, 4C): Apothecial morphology almost identical with those described for TRL-476, but possessed more light-colored disc (Brown, 6E7), and slightly more slender asci (47.0–76.0$\times$4.5–6.5 μm; $\bar{x}\pm$S.D.$=$67.5$\pm$8.1$\times$5.2$\pm$0.6 μm; n$=$13). Ascospore dimension (7.5–11.5$\times$2.0–2.5 μm; $\bar{x}\pm$S.D.$=$9.0$\pm$0.9$\times$ 2.0$\pm$0.2 μm; n$=$25) and morphology and dimension of ectal cells (11–17 μm in diam) were indistinguishable from those in TRL-476 (Fig. 4C).

Colony of SANK 19496 on PDA 29 mm in diam, characteristics similar to those of SANK 10294, but slightly different in having sectors where the surface becomes velvety.

TRL-131 (Figs. 2, 3E, F, 4A): Apothecia superficial, 0.8–1.4 mm in diam, seated on poorly developed subiculum-like hyphae which is hardly recognized, sessile, widely attached at the base; disc dull, smooth, Gray (2B1); receptacle black with well defined white margin, slightly elevated and irregularly incurving. Ectal excipulum of *textura globulosa*, composed of dark-brown isodiametric cells, 9.5–15 μm in diam. Asci 54.0–66.5$\times$4.5–6.0 μm ($\bar{x}\pm$S.D.$=$60.0$\pm$3.4$\times$5.5$\pm$0.6 μm; n$=$13), cylindrical, gradually tapered to the base, arising from croziers; apex hemispherical to slightly flattened, pore MLZ$+$ without KOH pretreatment. Ascospores 6.5–9.5$\times$2.5–3.0 μm ($\bar{x}\pm$S.D.$=$8.2$\pm$0.7$\times$2.7$\pm$0.2 μm; n$=$25), ellipsoid, occasionally somewhat curved, spumose when observed in MLZ. Paraphyses cylindrical, straight to slightly undulate, simple or branched near the base, septate, 3.0 μm in width, with obtuse apex.

Colony of SANK 19396 on PDA 23 mm in diam, low and dense, slightly umbonate at the center, Pale Yellow (3A3), inoculum Dark Brown (6F4), concolorous from the reverse, context tough and glutinous, sector and zonation absent. Aerial hyphae velvety to slightly floccose, white, forming weakly entangled strands at the center. Margin distinct, entire, superficial. Soluble pigment not produced. Anamorph not produced.

From the above mycological characteristics, all three isolates were thought to belong to the genus *Mollisia* (Fr.) P. Karst., Dermatiaceae, characterized mainly by dark-colored globular cells at the excipulum. The specimens TRL-476 and TRL-466 were thought to be conspecific (designated here as *Mollisia* sp. 1). TRL-131 was congeneric, but seemingly not conspecific with the above two (designated as *Mollisia* sp. 2). TRL-131 was distinguished from the other two in having more roundish ascospores (Q$=$3.0, while Q$=$4.75 in TRL-466 and 4.23 in TRL-476) (Fig. 4).

TRL-476 and TRL-466 were close to *Mollisia cinerea* (Batch: Fr.) P. Karst. Comparison with descriptions given for *M. cinerea* by Le Gal and Mangenot (1958) agreed well. However, characteristics were not decisive enough for identification. The genus *Mollisia* is very large, suspected to include some 100 species (Hawksworth et al. 1996), of which taxonomical studies

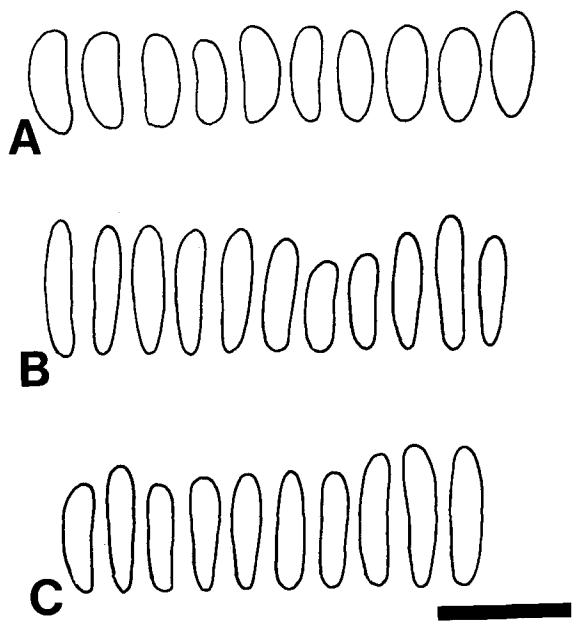

Fig. 4. Ascospores of TRL-131, 466 and 476.
A. TRL-131 (*Mollisia* sp. 2). B. TRL-476 (*Mollisia* sp. 1).
C. TRL-466 (*Mollisia* sp. 1).
Scale$=$10 μm.

are badly needed (Korf, 1973). Only two species have previously been reported in Japan: *M. cinerea* and *Mollisia albolaculans* Sydow (Otani, 1989), although *Mollisia* species are very common and many more species than previously reported must be distributed in Japan. There is no wide-ranging monographic literature available for identification. Due to this chaotic situation, the identification of specimens at the specific rank was left pending.

Discussion

This is the first report of production of zaragozic acids from discomycetes. ZAs are known to be distributed mainly among Pleosporales, but anticipated to be more widespread (Bills et al., 1994; Bergstrom et al., 1995). It is now clear that ZAs are distributed in all common traditional classes (Ainsworth, 1973) of ascomycetes, including discomycetes.

Bills et al. (1994) discussed the advantage of utilization of fungi characteristic of different substrata as a screening source of bioactive compounds. Most members of the discomycetes are not isolated from soil, but are frequently found on substrates other than soil, especially plant debris. Many of the conidial fungi on plant debris are, however, thought to be anamorphs of pyrenomycetes and loculoascomycetes. Anamorphs of discomycetes are known to be rare. So, discomycetes may be omitted in normal isolation procedures for conidial fungi both on wood and from soil. We isolated discomycetes with certainty by isolating ascospores from apothecia. A versatile approach for maximum ecological diversity was suggested by Bills (1995). The approach taken in the present study, therefore, can be interpreted as an attempt to take advantage of not only ecological but also phylogenetical differences. The usefulness of this kind of approach is recognized.

As previously discussed (Bills et al., 1994), there seems to be a relationship between taxonomy and the structure of the compound. ZA-As have been reported mainly from members of Pleosporales, traditionally classified in Loculoascomycetes, while other ZAs are reported from other classes of fungi. The major product of the three strains of *Mollisia* was ZA-D3, and ZA-A was not found. ZA-D3 has hitherto been reported from *Libertella* sp., which is thought to be an anamorph of pyrenomycetes (Bergstrom et al., 1995; Hawksworth et al., 1996).

On the other hand, Bills et al. (1994) discussed the possibility that biosynthesis of ZAs was conserved in evolutionary line on the basis of its identicality. The majority of the ZA-producing organisms reported belong to bitunicate ascomycetes. They are distantly related with unitunicate ascomycetes including Discomycetes, though they are thought to be derived from a common ancestor (Berbee, 1996). The hypothesis presented by Bills et al. (1994) implies the existence of related compounds even in distantly related groups of fungi. If the biosynthesis pathway in *Mollisia* spp. is also common with that previously reported, the pathway must have been acquired at an early stage of evolution and maintained since the common ancestor. It is also suggested that ZAs are basically important and common regulatory factors in metabolitic pathways in ascomycetes.

A limited number of isolates in certain species have superior productivity of secondary metabolites, though the given bioactive metabolites are distributed widely within a species. Concerning ZAs, ZA-B from *Sporormiella intermedia* is an example (Bills et al, 1994). Nearly all strains of *S. intermedia* tested had the capacity to produce ZA-B. As the production of F-10863s by *Mollisia* sp. 1 was found not to be restricted to a single isolate, this capacity may be widely present in *Mollisia* sp. 1. Although the production of all F-10863s was not confirmed in the small-scale fermentation in all three strains in the present study, large-scale fermentation may possibly provide some quantity of F-10863s.

Little attention has been paid to discomycetes as biological resources, due to their unfamiliarity in bioindustry and the difficulty of collecting them from nature. Discomycetes, however, are a relatively "easily accessible" (Bergstrom et al., 1995) resource, and Japan embraces a copious flora of these fungi. There are very few reports of bioactive compounds from *Mollisia* spp. at present, e.g., mollisin (van der Kerk and Overeem, 1957), and KS-504 (Nakanishi et al., 1989). But the biodiversity observed in the genus *Mollisia* suggests diversity of metabolites (Rossman, 1996). For utilization of these fungi, however, an overall inventory based on sound taxonomic studies will be needed.

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