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Discovery of dicephalosterol, a new testosterone 5α-reductase inhibitor, and some new mycological aspects of its producer, *Dicephalospora rufocornea* (Sclerotiniaceae, Discomycetes)

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Dicephalosterol, a new testosterone 5α-reductase inhibitor, was found from isolates of *Dicephalospora rufocornea*, a sclerotiniaceous discomycete widely distributed, but not previously cultured. Under SEM observation, the polar appendage of the ascospores in *D. rufocornea* was found to be more solid than was hitherto reported. Dicephalosterol was produced by submerged fermentation for 7 d. This new analogue of testosterone showed an IC_{50} of 5.7 μg/ml for rat prostatic 5α-reductase, but no antimicrobial activity against bacteria or fungi.

Key Words——bioactive metabolites; *Dicephalospora rufocornea*; dicephalosterol; microbial resources; testosterone 5α-reductase inhibitor.

Although a large number of discomycetes are cultivable, they have been less frequently isolated and their cultures have rarely been utilized as a screening source in discovery research for drugs, in contrast to soil-borne fungi (Hosoya, 1997, 1998). Given the wide diversity of physiological characters shown by discomycetes, such as metabolism and metabolites derived from their phylogenetic variation, it is tempting to expect novel metabolites from these underutilized resources. By pursuing this possibility, we have discovered new zaragozic acids from *Mollisia* spp. (Hosoya et al., 1997; Tanimoto et al., 1997). Since inoperculate discomycetes are widely distributed, frequently collected and cultivable, but not isolated from soils, we focused our attention on these discomycetes.

Inhibitors of testosterone 5α-reductase can be developed as a drug to prevent and/or cure prostatic hypertrophy, and some candidates for clinical application have already been found. Both testosterone analogues and non-analogues have been found (Brooks et al., 1986; Nakayama et al., 1989, 1990; Rasmusson et al., 1986). Some of the analogous compounds such as 4MA (*N,N*-diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide) have potent activity against 5α-reductase. Therefore, a search was undertaken for other analogues from microbial products. To reduce the possibility of rediscovering known compounds, frequently utilized microbial resources, mainly soil fungi, were laid aside.

In the screening program of testosterone 5α-reductase inhibitor using culture of discomycete isolates, several isolates of *Dicephalospora rufocornea* (Berkeley & Broome) Spooner (Sclerotiniaceae, Leotiales) showed certain activity. *Dicephalospora rufocornea* is distributed world-wide, particularly in the subtropics and tropics (Dumont, 1980). In spite of its wide distribution, there has been no cultural study of *D. rufocornea*. In the present paper, we report the discovery and structure of a new compound, designated dicephalosterol, which is responsible for testosterone 5α-reductase activity. We also report some mycological characters of *D. rufocornea* newly found in the present study.

Materials and Methods

Collection, isolation and observations for mycological characters Materials were collected in the Kanto area of Japan (Table 1). Collection and isolation methods followed Hosoya and Otani (1997a). The materials were identified based on morphological characteristics described by Dumont (1980), Otani (1990), and Spooner (1987). Single ascospore isolates were obtained in most cases, but some isolates were obtained from germinating spores in mass. Isolates were maintained on potato dextrose agar (PDA, Nissui, Tokyo) slants or on oatmeal agar (oatmeal 10 g, $MgSO_4 \cdot 7H_2O$ 1 g, KH_2PO_4 1 g, $NaNO_3$ 1 g, agar 20 g, and water 1 L) slants. Myco-

Table 1. List of *Dicephalospora rufocornea* isolates examined for production of dicephalosterol.

Isolate	origin*	collection site	collection date
Di-271	S	Yamatsuri ravine, Fukushima Pref.	July, 1991
F-9964	S	Mt. Tsukuba, Ibaraki Pref.	Oct., 1990
F-10753	M	Oharai, Ibaraki Pref.	June, 1990
F-12434	S	Hananuki ravine, Ibaraki Pref.	Nov., 1993
SANK 18291	S	Oharai, Ibaraki Pref.	June, 1990
SANK 10695	M	Kinoko-yama, Makabe, Ibaraki Pref.	Oct., 1990
SANK 10795	S	Mt. Tsukuba, Ibaraki Pref.	July, 1993

*S, originated from a single ascospore; M, originated from ascospores in mass.

logical characters were observed on PDA plates and oatmeal agar slants. SEM observation methods for ascospores followed Hosoya and Otani (1997b). Capitalized color indications followed Kornerup and Wanscher (1978). Growth in relation to temperature was observed in a temperature gradient incubator set at 0–50°C.

Small-scale fermentation For small-scale fermentation, 30 ml of the seed medium (soluble starch 20 g, glycerol 30 g, glucose 30 g, soybean meal 10 g, gelatin 2.5 g, yeast extract 2.5 g, NH_4NO_3 2.5 g, tap water 1 L) in a 100-ml Erlenmeyer flask was inoculated with mycelia of a slant culture. The seed culture was incubated at 23°C for 1 wk on a rotary shaker operating at 210 r.p.m. 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 previously described. The culture was extracted with equal volume of acetone. After filtration to discard mycelia, acetone was evaporated. The remaining solution was extracted with equal volume of ethyl acetate in acidic conditions (pH 3.0) to result in an extract of 10 X concentration.

Assays Assay of rat prostatic 5α-reductase inhibition activity followed Kurata et al. (1996). Antimicrobial activity of dicephalosterol at 1.0 mg/ml was assayed using the disc assay technique aginst bacteria (*Bacillus subtilis* (Ehrenberg) Cohn, *Bacteroides fragilis* (Veillon & Zuber) Castellani & Chalmers, *Enterococcus faecalis* (Andrewes & Horder) Schleifer & Kilpper-Baelz, *Escherichia coli* (Migula) Castellani & Chalmers, *Klebsiella pneumoniae* (Schroeter) Trevisan, *Mycobacterium smegmatis* (Trevisan) Lehmann & Neumann, *Mycoplasma mycoides* (Borrel et al.) Freundt, *Proteus mirabilis* Hauser, *P. vulgaris* Hauser, *Pseudomonas aeruginosa* (Schroeter) Migula, and *Staphylococcus aureus* Rosenbach) and fungi (*Aspergillus niger* van Tieghem, *Candida albicans* (Robin) Berkhout, *Mucor hiemalis* Wehmer, and *Trichophyton mentagrophytes* (Robin) Blanchard).

Large-scale fermentation To obtain a larger quantity of dicephalosterol, the strain SANK 10695 was used. A 500-ml Erlenmeyer flask containing 100 ml of culture medium (glucose 1 g, glycerol 1 g, sucrose 1 g, soybean meal 2 g, oatmeal 0.5 g, casamino acid 0.5 g, $CaCO_3$ 0.1 g, tap water 100 ml) was inoculated with mycelia from a slant culture and cultivated at 23°C for 7 d at 210 r.p.m. on a rotary shaker.

Portions of this culture broth (3 ml each) were inoculated into 50 500-ml Erlenmeyer flask each containing 100 ml of medium composed of glycerol 70 g, polypeptone 10 g, soybean meal 10 g, $MgSO_4 \cdot 7H_2O$ 1 g, glucose 30 g, $NaNO_3$ 5 g, CSL 10 g, tap water 1 L. These flasks were then subjected to shaking culture at 23°C for 7 d at 210 r.p.m.

Isolation of dicephalosterol Acetone (5 L) was added to the culture. After filtration to discard mycelia, acetone was evaporated. The remaining solution was fractionated by HP-20 column chromatography (500 ml, acetone-H_2O 0 : 1–1 : 0) to give an oily substance. This oily material was subjected to silica gel column chromatography (hexane-ethylacetate 2 : 8–0 : 10), and further purified by silica gel column chromatography (hexane-ethylacetate 2 : 8) to give dicephalosterol.

Structure elucidation Structure of dicephalosterol was determined by ^1H, ^{13}C, and MS spectroscopy. The details of structure determination will be discussed elsewhere.

HPLC detection of dicephalosterol An equal amount of acetone was added to the culture. Following filtration, acetone was evaporated. The remaining solution was fractionated by HP-20 column chromatography (500 ml, acetone-H_2O 0 : 1–1 : 0) to give an oily substance. This oily substance was subjected to HPLC (Symmetry C18, 4.6 mm × 150 mm, Waters; 50% aquaeous acetonitrile, 1 ml/min.) to detect dicephalosterol. Dicephalosterol was detected at a retention time of 5.0 min.

Results

Taxonomy and mycological characters Morphological characters of the apothecia from which each isolate was obtained agreed well with previous description of *D. rufocornea* (Dumont, 1980; Otani, 1990; Spooner, 1987). The polar appendages of the ascospores were observed in greater detail than previously under SEM (Fig. 1) and found to be non-membranaceous, solid, and determinate in structure.

The cultural characters were as follows (Fig. 1). Colonies on PDA attaining a diameter of 2 cm in 10 d at 25°C; plane, velvety, later funiculose, floccose at the center due to the development of aerial hyphae; in

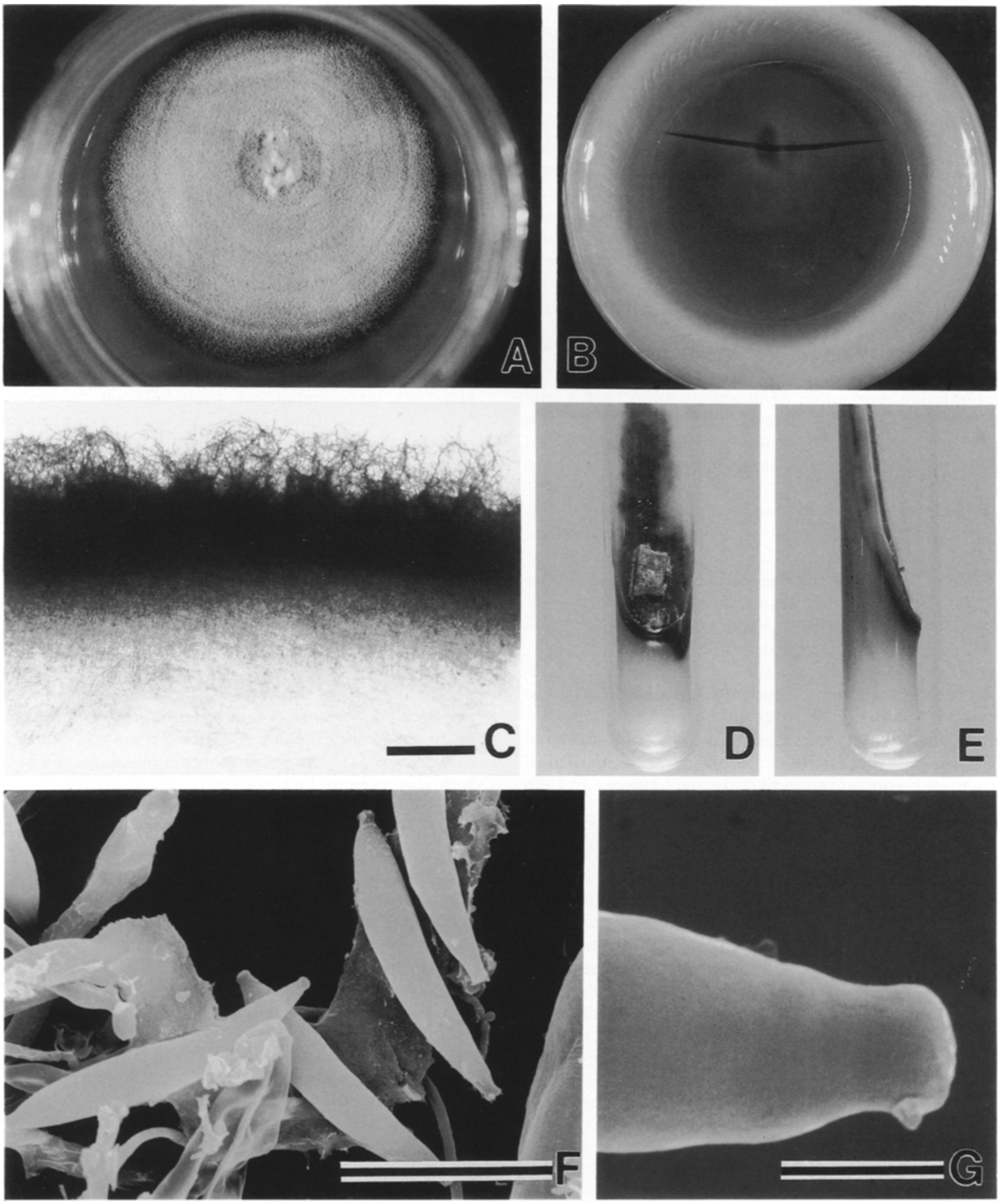

Fig. 1. Mycological cahracters of *Dicephalospora rufocornea*. A. Colony morphology of SANK 10695 on PDA (2 mo, 23°C) from the surface. B. Colony morphology of SANK 10695 on PDA (2 mo, 23°C) from the reverse. C. Mat-like structure observed in section of a colony formed on PDA (2 mo, 23°C). D. Slant culture of SANK 18291 on oat-meal agar (3 mo, 23°C) viewed from the front. E. Slant culture of SANK 18291 on oat-meal agar (3 mo, 23°C) viewed from the side. Note black mat-like structure formed beneath the surface of the medium. F. Ascospores observed under SEM. Note polar appendages. G. Close-up view of the ascospore polar appendage.
Scales: C, 500 μm; F, 20 μm; G, 2 μm.

prolonged incubation up to 2 mo becoming Reddish Grey (9B2) with marginal area of Reddish Brown (8F8) from the surface, Reddish Brown (8D8) from the reverse due to the excretion of dark orange soluble pigments into agar. Margin entire. Context tough and gelatinous. In section, dark-colored mycelial mat of 700–800 μm in thickness, developing beneath the surface of the medium, composed of tightly entangled, darkened hyphae. In prolonged incubation (3 mo) on oatmeal agar slant, a black layer similar to that on PDA was formed along the surface of the agar. Differentiation of rind and medulla not clearly observed. Neither sclerotia nor conspicuous linear structures delimiting stroma observed even on prolonged incubation. No conidia producing structures observed.

Growth occurred at 10–32°C, optimal at 23–29°C; pigment production abundant over 20°C.

Fermentative production of dicephalosterol A 5-L culture of the strain SANK 10695 yielded 2.74 g of brown oily material after HP-20 column chromatography. After purification by silica gel column chromatography 65.6 mg of colorless needles of dicephalosterol was obtained. Dicephalosterol was detected in small-scale fermentation samples of six other isolates by HPLC, but not exactly quantified.

Physico-chemical properties of dicephalosterol The physico-chemical properties of dicephalosterol are summarized in Table 2. The structure of dicephalosterol was found to be analogous to testosterone (Fig. 2).

Biological activity All the samples produced by small-scale fermentation of *D. rufocornea* isolates inhibited rat prostatic 5α-reductase. Purified dicephalosterol inhibited rat prostatic 5α-reductase with the IC_{50} value of 5.7 μg/ml. Dicephalosterol did not show antimicrobial activity at a concentration of 1.0 mg/ml against any the organism tested.

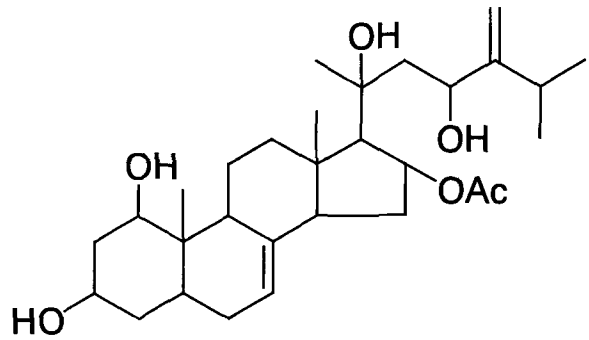

Fig. 2. Structure of dicephalosterol.

Discussion

The distribution of *D. rufocornea* on various substrates in southern Japan has been indicated by Dumont (1980) and Otani (1990). Dumont (1980) first found stroma at the base of apothecia, and transferred the species from Leotiaceae to Sclerotiniaceae as a species of *Lanzia* Sacc. Spooner (1987) proposed the new generic name *Dicephalospora* Spooner for the present fungus based on the presence of polar appendages. Dumont (1980) reported that stroma of *D. rufocornea* in the natural substrata were sometimes being difficult to observe. Many members of the Sclerotiniaceae form conspicuous sclerotia or stromatic structures in culture. Although a mat-like structure was formed in *D. rufocornea* in the present study, a conspicuous stromatic structure was not present. Dumont (1980) and Otani (1990) noted that the stroma of *D. rufocornea* was variable in form and difficult to observe. The diffuse mat-like structure formation may reflect its indeterminate stroma formation in nature.

The polar appendages of *D. rufocornea* have long been overlooked. Dennis (1963) failed to observe the appendages. Dumont (1980) noted them as "flarings", and found that the appendage is not present in all collections. Spooner (1987) first interpreted the appendages as an important criterion, describing them as "mucilaginous collars". In the present study, however, the structure was found to be more solid. Spore appendages are usually thought to help adhesion of ascospores (Jones, 1994), but the function of appendages in *D. rufocornea* is not clear.

The optimal temperature for growth of around 25°C, and ability to grow at over 30°C coincide with the occurrence of *D. rufocornea* in tropical area. In the first author's experience, discomycetes with optimal growth temperatures of over 25°C have not frequently been encountered in Japan.

The structure of dicephalosterol was a new analogue of testosterone. Previously discovered inhibitors of

Table 2. Physico-chemical characters of dicephalosterol.

mp.: 152°C
Molecular formula: $C_{30}H_{48}O_6$ [HRFAB-MASS *m/z* 505.3526, Δ −0.6 mmu $(M+H)^+$]
IR (KBr): 3490, 3370, 2960, 2930, 1730, 1710, 1370, 1270, 1040, 1030 cm^{-1}
^1H NMR (δ) ppm 5.13 (1H, brt J=6.6 Hz), 5.13 (1H, brs), 5.05 (1H, s), 4.88 (1H, s), 4.47 (1H, brd J=10.2 Hz), 3.92 (1H, m), 3.78 (1H, brs), 2.16–2.26 (3H, m), 1.97 (3H, s), 1.94–1.97 (2H, m), 1.46–1.81 (12H, m), 1.44 (3H, s), 1.25–1.29 (1H, m), 1.08 (3H, d J=6.9 Hz), 1.06 (3H, d J=6.9 Hz), 0.79 (3H, s). ^{13}C NMR (δ) ppm 172.8 (s), 161.4 (s), 140.5 (s), 119.3 (d), 108.0 (t), 77.9 (d), 76.6 (s), 73.7 (d), 73.2 (d), 67.9 (d), 66.8 (d), 54.4 (d), 48.0 (t), 46.3 (t), 42.3 (d), 42.1 (t), 39.9 (s), 39.7 (t), 39.3 (t), 35.5 (d), 35.1 (t), 31.5 (t), 26.6 (q), 24.7 (q), 24.1 (q), 22.0 (q), 21.8 (t), 15.5 (q), 14.5 (q).
UV: end absorption

testosterone 5α-reductase are testosterone analogues (Brooks et al., 1986; Rasmusson et al., 1986). The absence of anti-microbial activity suggests its specificity for 5α-reductase inhibition.

Production of dicephalosterol is suggested to be species-specific, rather than isolate-specific. Although dicephalosterol is produced in low amounts in culture, it may be involved in the regulation of sterol metabolism.

Discomycetes are underutilized but easily accessible microbial resources (Bergstrom et al., 1995). The present study represents another successful example of utilization of previously uncommon microbial resources to discover useful bioactive metabolites.

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